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Poxvirus vaccine strategies to improve T cell responses: neutrophil immunomodulation & promoter modification

Tesis doctoral de Mauro Di Pilato

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CERTIFICA

Que la Tesis Doctoral titulada "**Poxvirus vaccine strategies to improve T cell responses: neutrophil immunomodulation & promoter modification**" ha sido realizada en el Centro Nacional de Biotecnología y tutelada en el Departamento de Biología Molecular de la Universidad Autónoma de Madrid.

El trabajo realizado por Mauro Di Pilato reúne todas las condiciones requeridas por la legislación vigente, así como la originalidad y calidad científica para poder ser presentada y defendida con el fin de optar al grado de Doctor.

Y para que conste donde proceda, firmo el presente certificado

Madrid a 6 de Abril de 2015

Prof. Mariano Esteban Rodríguez
Director de la Tesis

Ai **miei genitori**,
che mi hanno lasciato volare,
e questo mio bel volo
è frutto dei loro sacrifici.

A **Samuel** e a mio **nonno Mauro**,
le cui vite si sono incrociate solo per un anno,
ma che sono accumunati
dall'essere i miei due più grandi tifosi.

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che non sono stati qui durante questo racconto,
ma che ci sono sempre
e sempre ci saranno.

Loro non si arrenderanno mai
(ma gli conviene?),
noi neppure.
Cit. M5S

A veces
la persona que nadie imagina capaz de nada
es la que hace cosas que nadie imagina.
Cit. Imitation game

Luck is an attitude.
Cit. Martini

La goccia disse alla roccia:
"Timbe ce vole,
ma u buche t'u a ià fa!"
Cit. Anonimo Barese

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Presentación

El virus vaccinia está siendo utilizado como vector vacunal en varias enfermedades animales y humanas. La capacidad de inducir una respuesta de células T específicas fretne a los antígenos exógenos constituye la mejor propiedad inmune de este virus que genera protección frente a distintos patógenos. MVA y NYVAC, con capacidad reducida de replicación en las células humanas, representan los vectores más utilizados del virus vaccinia en ensayos clínicos.

Este trabajo se dirige hacia la comprensión de los mecanismos involucrados en la generación de respuestas inmunes específicas hacia antígenos y a la mejora de la capacidad de inducir esta respuesta por parte de los poxvirus. En este sentido, hemos desarrollado diferentes estrategias y distintos candidatos vacunales del virus vaccinia para mejorar las respuestas inmunes específicas hacia antígenos heterólogos.

Hemos modificado el vector NYVAC-C, que expresa antígenos del virus VIH-1 de subtipo C, mediante deleción de genes (*A52R*, *K7R*, *B15R*) para enfocarnos en la vía de señalización de NFκB en la célula huésped. Se encontró que estos genes actúan conjuntamente para inhibir la vía de señalización de NFκB, descartando la sinergia entre estas proteínas como modo de acción. Se estudió la capacidad de este virus recombinante de inducir respuestas inmunes innatas que dependen de NFκB y se demostró que los neutrófilos aumentan las respuestas inmunitarias adaptativas de células T hacia antígenos de VIH. Definimos el tráfico de neutrófilos desde el sitio de infección hacia diversos órganos linfoides secundarios y describimos que después de la estimulación por quimiocinas, los neutrófilos polarizan hacia fenotipos funcionales distintos que regulan la magnitud y la calidad de la respuesta inmune.

Hemos generado nuevos promotores del virus vaccinia para mejorar la expresión de los antígenos GFP y LACK de *Leishmania* y aumentar las respuestas de células T específicas hacia estos antígenos. Tras el análisis bioinformático, se definió una nueva secuencia del promotor para mejorar los niveles de expresión temprana del antígeno GFP. Por otra parte, hemos modificado la longitud del espaciador del promotor para aumentar la expresión de estos dos antígenos heterólogos. Los vectores recombinantes de MVA con estas modificaciones en el promotor aumentaron la expresión del gen heterólogo y positivamente influyeron en la magnitud y la calidad de las respuestas de memoria de células T específicas. Estos resultados proporcionan importantes conocimientos sobre el mecanismo de la respuesta inmune inducida por el virus vaccinia y estrategias alternativas para el diseño de vectores vacunales.

Summary

Vaccinia virus (VACV) is being used as vaccine vector for several animal and human diseases. Its capacity to induce specific T cell responses to foreign antigens define the best immune property of this virus, which generates protection against distinct pathogens. The MVA and NYVAC virus strains, which have limited replication in human cells, are the most-used VACV vector in clinical trials.

This study is directed to understanding the poxvirus-dependent mechanisms involved in the generation of antigen-specific immune responses, and to improving poxvirus capacity to induce this response. Here we developed different strategies and distinct VACV-based vaccine candidates to improve foreign antigen-specific immune responses. We modified NYVAC-C that expressed HIV-1 clade C antigens by gene deletion (*A52R*, *K7R*, *B15R*) to target the NFκB central host-cell signaling pathway. We found that these genes act together to inhibit NFκB signaling, ruling out synergy between these proteins as a mode of action. Our study of the ability of this recombinant virus to induce NFκB-dependent innate immune responses demonstrated that neutrophils increase the T cell adaptive immune response to HIV antigens. We tracked the neutrophil trafficking from the infection site to various secondary lymphoid organs and showed that, after chemokine stimulation, neutrophils polarize to distinct functional phenotypes that regulate the magnitude and quality of the immune response.

We generated new VACV promoters to improve the timing of GFP (green fluorescent protein) and LACK (*Leishmania* homologue of receptors for activated C-kinase) expression and to increase antigen-specific T cell responses. Using bioinformatics analysis, we defined a new early promoter motif to improve GFP antigen early expression. Furthermore, we modified the promoter spacer length to increase this heterologous antigen expression. The recombinant MVA vectors with these promoter modifications increased heterologous gene expression and positively influenced the magnitude and the quality of antigen-specific T cell memory responses.

These findings provide important insights into the mechanism of the VACV-induced immune response and alternative strategies for vaccine vector design.

Index

ABBREVIATIONS

A

AIDS: acquired immune deficiency syndrome

AP-1: activator protein-1

APC: antigen presenting cells

B

Bcl-2: B-cell lymphoma 2

C

CCR1: chemokine (C-C motif) receptor 1

CCR2: chemokine (C-C motif) receptor 2

CCR5: chemokine (C-C motif) receptor 5

CD: cluster of differentiation

CEV: cell-associated enveloped virus

CVA: chorioallantois vaccinia virus Ankara

D

DC: dendritic cells

DNA: deoxyribonucleic acid

E

EEV: extracellular enveloped virus

Env: envelope protein

ETF: early transcription factor

EV: enveloped virion

G

GFP: green fluorescent protein

GLA: glucopyranosyl lipid adjuvant

GM: granulocyte macrophage-colony-stimulating factor

GP120: glycoprotein 120

GPN: Gag-Pol-Nef

H

mH5: modified H5

HIV: human immunodeficiency virus

I

ICTV: International Committee on Taxonomy of Viruses

IEV: intracellular enveloped virus

IFN: interferon

IFN-α: interferon alpha

IFN-γ: interferon gamma

IKK: IκB kinase

IL-1β: interleukin 1 beta

IL-2: interleukin 2

IL-4: interleukin 4

IL-6: interleukin 6

IL-8: interleukin 8

IL-10: interleukin 10

IL-13: interleukin 13

IMV: intracellular mature virus

IRF 3: IFN-regulatory factor 3

IRF 7: IFN-regulatory factor 7

IRAK: IL-1R-associated kinase

L

LACK: *Leishmania* homologue of receptors for activated C kinase

LEO: late-early optimized

LEO99: late-early optimized 99

LEO160: late-early optimized 160

dLN: draining lymph nodes

M

Mϕ: macrophage

MAL: MyD88-adaptor-like

MCP-1/CCL2: monocyte chemoattractant protein-1

MEME: Multiple EM for Motif Elicitation

MHC-II: major histocompatibility complex type II

MIP 1α/CCL3: macrophage inflammatory protein 1 alpha

MIP-1β/CCL4: macrophage inflammatory protein 1 beta

MV: mature virion

MVA: modified vaccinia virus Ankara

MyD88: myeloid differentiation factor 88

N

Nα: neutrophil alpha

Nβ: neutrophil beta

NET: neutrophil extracellular trap

NFκB: nuclear factor kappa-light-chain-enhancer of activated B cells

NK: natural killer

NLR: NOD-like receptor

NOD: nucleotide-binding oligomerization domain

nt: nucleotide

NYVAC: New York vaccinia virus

O

ORF: open reading frame

P

PAMP: pathogen-associated molecular pattern

PRR: pattern recognition receptor

pS: synthetic promoter

R

RAP94: RNA polymerase-associated protein 94

RIG: retinoic acid inducible gene

RLR: RIG like receptor

RNA: ribonucleic acid

mRNA: messenger RNA

S

SIV: simian immunodeficiency virus

T

TAB1: TAK1-binding protein 1

TAB2: TAK1-binding protein 2

TAN: tumor-associated neutrophil

TAK1: TGF-β-activated kinase 1

TGF-β: transforming growth factor beta

Th: T helper cell

TIR: Toll/IL-1 receptor

TLR: Toll-like receptor

TNF: tumor necrosis factor

TNFα: tumor necrosis factor alpha

TRAF 6: TNF receptor-associated factor 6

TRAM: TRIF-related adaptor molecule

TRIF: TIR domain-containing adapter inducing IFNβ

V

VACV: vaccinia virus

INTRODUCTION

1.1. Poxvirus

Poxviruses are enveloped DNA viruses that replicate in the cell cytoplasm and their complex morphogenesis includes the *de novo* synthesis of virus-specific membranes (Buller and Palumbo, 1991). According to the International Committee on Taxonomy of Viruses (ICTV), the Poxviridae family is divided into two subfamilies, the *Entomopoxvirinae* and the *Chordopoxvirinae*, which infect invertebrates and vertebrates, respectively. *Orthopoxvirus* includes species isolated from mammals and is one of the ten *Chordopoxvirinae* genera. Although variola virus, the causative agent of smallpox, is the most famous member of the genus, the prototype vaccinia virus (VACV) is the most studied specie.

1.1.1. Vaccinia virus

Vaccinia virus (VACV) has a brick-shaped structure with fixed dimensions of 360 x 270 x 250 nm (Cyrklaff et al., 2005) (Figure 1A). Infectious VACV particles present two forms; the mature virion (MV) is composed by a nucleoprotein core, inclusion bodies, structural proteins and enzymes used for the early gene transcription, surrounded by a lipoprotein membrane (Condit et al., 2006), whereas the enveloped virion (EV) has six additional proteins associated to an antigenically distinct outer membrane that is generated by fusion with the host cell membrane (Smith et al., 2002). These six additional proteins are involved in virus entry into host cells and immune evasion (Payne, 1978) (Figure 1B).

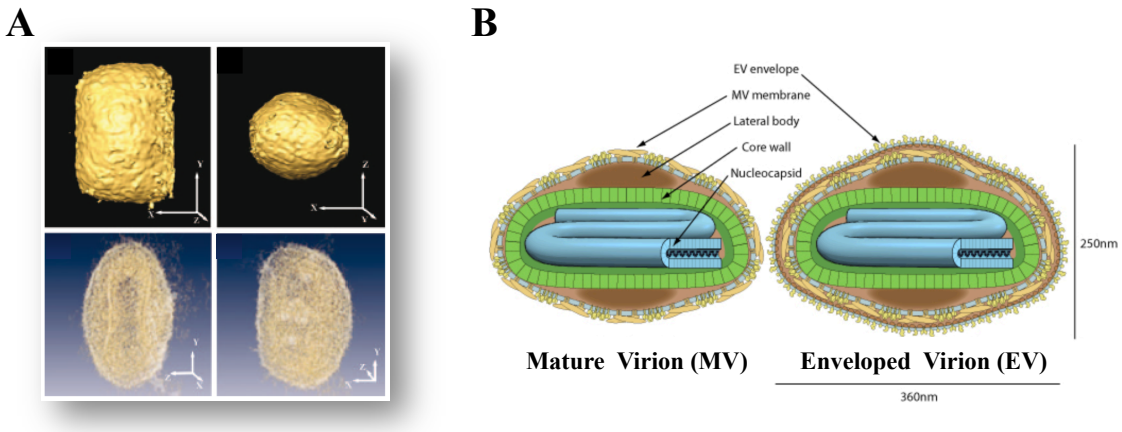

Figure 1. VACV structure and morphology

(A) Cryo-electron tomography of vaccinia virus (Cyrklaff et al., PNAS, 2005).

(**B**) Structure of infectious vaccinia virus particles (Swiss Institute of Bioinformatics, 2008).

VACV has a double-stranded DNA genome of 62.3 μm (Esteban et al., 1977) and ~200 kb, which encodes 250 genes (Goebel et al., 1990). The gene open reading frames (ORF) are named according to size in kb, location, position, and direction of transcription (L, left; R, right) within the HindIII digestion sites (Roseman and Slabaugh, 1990). The most-conserved genes are located centrally and are involved in replication or virion assembly, and most-variable genes are located terminally and participate in host range restriction or immune evasion (Seet et al., 2003).

The VACV life cycle (Figure 2) begins with virion entry into the cell by fusion with the plasma membrane or by endocytic uptake (macropinocytosis) and with the core release into the host cytoplasm (McFadden, 2005). After core uncoating, VACV itself carries on its early gene transcription. The early mRNA are translated into early proteins involved in DNA replication, intermediate transcription and host immune evasion (McFadden, 2005). The intermediate proteins participate in the late transcription of proteins involved in virus assembly. During morphogenesis, core protein and condensed DNA enclosed by a membrane form the intracellular mature virus (IMV) (McFadden, 2005). IMV particles assemble and migrate via microtubule-mediated trafficking and are then wrapped with Golgi-derived membranes to form intracellular enveloped virus (IEV) (McFadden, 2005). IEV can nucleate the formation of actin tails that aid its movement to the cell surface (Cudmore et al., 1995). IEV can fuse with the plasma membrane to form the cell-associated enveloped viruses (CEV) that remain attached to the cell and are involved in cell-to-cell spread, as well as the extracellular enveloped viruses (EEV) that are shed by the cell and mediate long-range virus dissemination (Blasco and Moss, 1991).

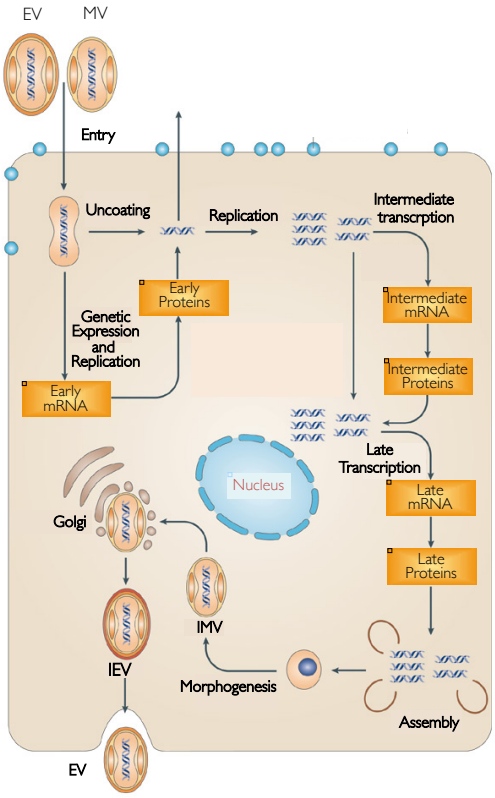

Figure 2. VACV life cycle
(adapted from McFadden et al. *Nat Rev Microbiol* 2005).

1.1.2. VACV as a vaccine vector

VACV was used as a vaccine to eradicate smallpox (Wehrle, 1980); several later studies demonstrated that VACV vector can stably incorporate different exogenous DNAs and induces protection against distinct pathogens (Moss, 1991; Paoletti, 1996). VACV has unique vaccine features (Pastoret and Vanderplasschen, 2003):

- High stability of the freeze-dried vector
- Low production cost
- Easy manufacture and production
- Ability to incorporate large amounts (up to 25 kb) of DNA
- High heterologous antigen expression
- Good capacity to induce cellular and humoral responses

The safety issues, due to some adverse reactions in VACV vaccination programs (Lane et al., 1969; Redfield et al., 1987), led to the generation of attenuated VACV such as MVA and NYVAC, that have limited replication in human cells (Moss, 1996).

1.1.3. Modified vaccinia virus Ankara (MVA)

MVA was derived from the chorioallantois vaccinia Ankara (CVA) strain after 570 passages on chicken embryo fibroblasts (Hochstein-Mintzel et al., 1975), resulting in the loss of ~15% (30 kb) of the parental viral genome (Antoine et al., 1998) (Figure 3A); the deleted genes are involved in virulence and host immune evasion (Meyer et al., 1991). MVA expresses early, intermediate and late proteins, but does not carry out virion assembly during morphogenesis in HeLa cells (Sancho et al., 2002).

MVA, which generates an immune response more rapidly than fully replication-competent viruses (Earl et al., 2008), is widely studied as a new generation of poxvirus vaccine (McCurdy et al., 2004) for its capacity to induce protection in mice and in non-human primate models (Stittelaar et al., 2005; Wyatt et al., 2004). MVA has been recently tested as a vaccine candidate in several clinical trials for prevention and treatment of human diseases such as AIDS (acquired immune deficiency syndrome) (Keefer et al., 2011; Kibler et al., 2011), hepatitis (Fournillier et al., 2013), malaria (de Barra et al., 2014), tuberculosis (Meyer et al., 2013) and cancer (Harrop et al., 2006).

1.1.4. New York vaccinia virus (NYVAC)

NYVAC was derived from the Copenhagen vaccine strain by the precise deletion of 18 open reading frames (ORF) from the parental viral genome (Tartaglia et al., 1992); the deleted genes (15 kb) are involved in pathogenicity, virulence and host-range regulatory functions (Tartaglia et al., 1992) (Figure 3B). In HeLa cells, through a transcription blockade, NYVAC does not express certain late proteins that participate in morphogenesis (Tartaglia et al., 1992).

NYVAC has been studied for its ability to induce good antigen-specific immune responses in the mouse and non-human primates (Flynn et al., 2011; Gomez et al., 2007c), and has been tested as a human immunodeficiency virus (HIV) vaccine candidate in clinical trials (Bart et al., 2014; Harari et al., 2012).

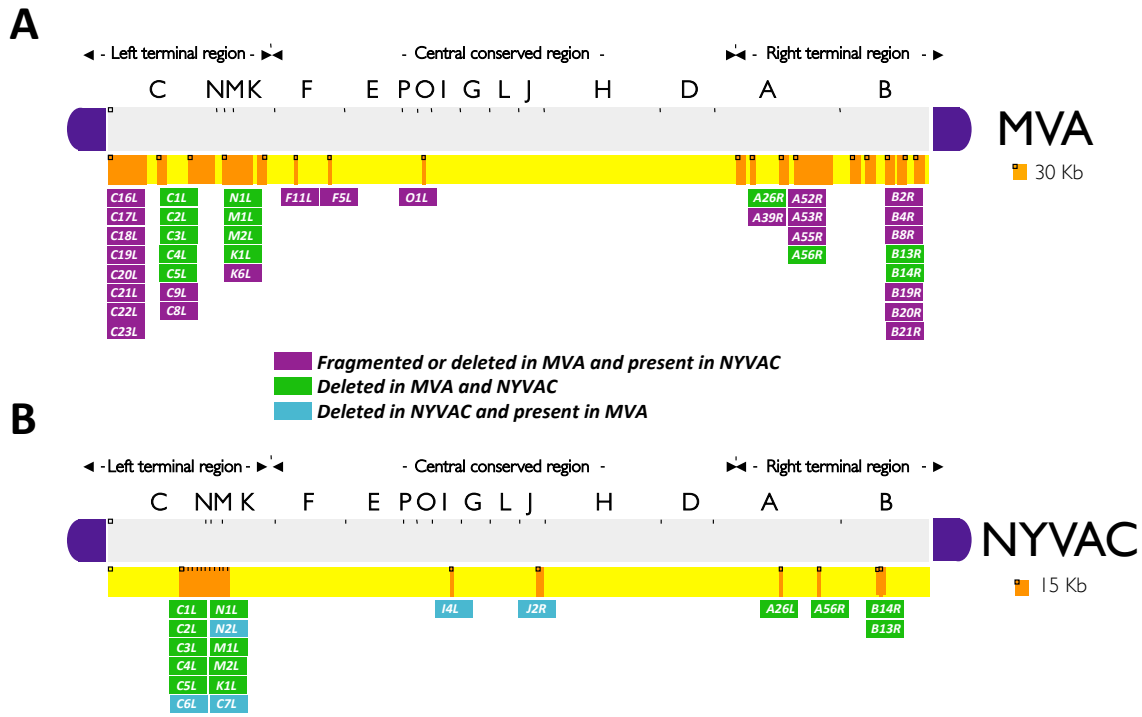

Figure 3. VACV genomes

(A) MVA genome, **(B)** NYVAC genome (adapted from Gomez et al., *Curr Gene Ther*, 2011).

Comparison of MVA and NYVAC *in vivo* dissemination showed that both viruses reach and infect target tissues, although with different kinetics; MVA induces a faster antiviral response and viral clearance than NYVAC, which expresses viral genes for 72 h post-infection, 24 h more than MVA (Gomez et al., 2007a). Several microarray studies demonstrated that MVA and NYVAC infection regulate distinct gene expression

profiles in human cells (Guerra et al., 2004; Guerra et al., 2006; Guerra et al., 2007), which influences virus capacity to escape the immune system (Gomez et al., 2008). Variation in virus capacity to activate the innate signaling pathway and to control viral gene expression is responsible for generation of different T cell responses (Obst et al., 2005); specifically, MVA elicited more CD8 and less CD4 T cells responsive to HIV antigens than NYVAC in a primate model (Mooij et al., 2008).

1.1.5. MVA and NYVAC vaccine strategies to increase antigen immunogenicity

Attenuated VACV vectors such as MVA and NYVAC are being tested as candidate vaccines to express heterologous pathogen antigens, and are being further improved to potentiate antigen-specific immune responses (immunogenicity) (Garcia-Arriaza and Esteban, 2014). The distinct levels of immunogenicity of all antigens expressed by a virus define the viral immunodominance hierarchy (Yewdell and Bennink, 1999). Immunodominance is the phenomenon whereby only a small fraction of all of possible pathogen epitopes (the part of the antigen recognized by the immune system) elicits an immune response in an infected individual (Pasquetto et al., 2005). During infection in specific T cell immune conditions such as pre-priming of T cells or regulatory T cell depletion (Assarsson et al., 2007), subdominant epitopes, which are immunogenic and generated by natural antigen processing but not normally recognized by immune responses (Franke et al., 2000), can break this hierarchy at the expense of dominant epitopes. Viral antigen uptake (Siddiqui and Basta, 2011), the timing and quantity of its expression (Wilson and Hunter, 2008), its cellular transport (Li et al., 1997), and/or its processing/proteolysis (Jing et al., 2007; Pamer and Cresswell, 1998) can positively influence its immunogenicity. The VACV vectors and/or the route of infection could also influence the immunodominance hierarchy (Tscharke et al., 2005).

Several strategies have been developed to improve immunogenicity to heterologous antigens expressed by MVA and NYVAC vectors (Garcia-Arriaza and Esteban, 2014; Gomez et al., 2012a, 2013):

- heterologous prime/boost immunization protocols such as DNA-MVA, DNA-NYVAC, MVA-NYVAC, NYVAC-MVA, MVA-MVA and NYVAC-NYVAC protocols to selectively increase the antigen-specific T cell response (Boukhebza et al., 2012; Gomez et al., 2007b)

- use of MVA virus with costimulatory molecules and cytokines such as CD40L (Gomez et al., 2009) or IL-2 (Ramlau et al., 2008) to directly induce T cell activation and proliferation
- insertion of host range viral genes such as *K1L* (Kovarik et al., 2001), *C7L* (Najera et al., 2010), or both (Kibler et al., 2011; Mooij et al., 2015) to generate attenuated replication-competent vectors that guarantee higher antigen expression
- insertion of synthetic or natural early promoters (Baur et al., 2010; Isshiki et al., 2014; Sato et al., 2013; Wennier et al., 2013) to increase the quality of antigen expression
- deletion of immunodulatory genes that encode inhibitors of apoptosis pathways such as F1 (Perdiguero et al., 2012), interferon (IFN) receptor-mediated signals such as B8 and B19 (Gomez et al., 2012b), Toll-like receptor (TLR) pathways such as C6 (Garcia-Arriaza et al., 2011) or N2 (Garcia-Arriaza et al., 2014), to increase antigen uptake by the innate immune system
- use of MVA virus with adjuvants such as IC31 (Pattacini et al., 2012), TLR 9 agonist, or the TLR 4 agonists A27 fusion protein (Vijayan et al., 2012) and glucopyranosyl lipid adjuvant (GLA) (McKay et al., 2014), to increase antigen recognition in the innate immune response.

1.2. Antiviral innate immunity

Pathogen-associated molecular patterns (PAMP) such as viral protein and genomic DNA or RNA induce the innate immune response by activation of host cell pattern recognition receptors (PRR) (Smith et al., 2013). The PRR include membrane-associated TLR, retinoic acid inducible gene I (RIG-I)-like receptors (RLR), nucleotide-binding oligomerization domain (NOD)-like receptors (NLR) and cytoplasmic DNA sensors (Perdiguero and Esteban, 2009). NLR regulate interleukin (IL)-1β maturation (Kanneganti et al., 2007), whereas TLR and RLR are involved in the transcription of type I interferon (IFN) cytokines and chemokines (Smith et al., 2013). This gene transcription is mediated by IFN-regulatory factor (IRF) 3, nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), and activator protein 1 (AP-1), which form an enhanceosome complex that binds to the IFN-β promoter (Wathelet et al., 1998); the IRF-7 transcription factor also promotes IFN-α gene expression (Marie et al., 1998).

1.2.1. TLR signaling pathway and NFκB activation

NFκB pathway activation depends on virus capacity to interact with TLR. Viral envelope proteins interact with TLR2 and TLR4 on the plasma membrane (Akira et al., 2006), while RNA and DNA recognize endosomic TL3/7/8 and TLR9, respectively. PAMP binding induces TLR2 and TLR4 homo- or heterodimerization (Jin and Lee, 2008), followed by recruitment of Toll/IL-1 receptor (TIR) domain-containing adaptor proteins such as myeloid differentiation factor 88 (MyD88) and MyD88-adaptor-like (MAL) or TIR domain-containing adapter inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM). MyD88 mediates the activation of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) by phosphorylation of IL-1R-associated kinase (IRAK) 1,2 and 4; TRIF interacts directly with TRAF6. TRAF6 recruits transforming growth factor (TGF)-β-activated kinase (TAK1) as well as TAK1-binding proteins 1 (TAB1) and 2 (TAB2) to phosphorylate TAK1 and to activate the IκB kinase (IKK) complex; this activation leads to IκBα phosphorylation and subsequent degradation mediated by proteasome, allowing release of the NFκB complex (comprised of p65/p50 or p65/p52 heterodimers) into the cell nucleus. NFκB then binds the κB site and regulates transcription of pro-inflammatory cytokine and chemokine genes (Akira et al., 2006).

1.2.2 VACV and TLR/NFκB pathway

The VACV envelope interacts with TLR2 and TLR4 (Delaloye et al., 2009; Hutchens et al., 2008), and produces early viral inhibitors of the NFκB pathway (Figure 4) such as:

- A46, which disrupts the TLR4 receptor:TIR adaptor link (Stack et al., 2005)
- A49, which inhibits IκBα degradation (Mansur et al., 2013)
- A52, which blocks IRAK2 and TRAF6 activation (Harte et al., 2003)
- B14 (B15 in NYVAC), which impedes IκBα phosphorylation (Chen et al., 2008)
- C4, which inhibits NFκB activation downstream of IKK (Ember et al., 2012)
- E3, which sequesters RNA to avoid binding with PRR (Myskiw et al., 2009)
- K1, which prevents IκBα degradation (Shisler and Jin, 2004)
- K7, which blocks IRAK2 and TRAF6 activation (Schroder et al., 2008)
- M2, which avoids NFκB translocation to the nucleus (Gedey et al., 2006)
- N1, which inhibits IKK activation (Maluquer de Motes et al., 2011)

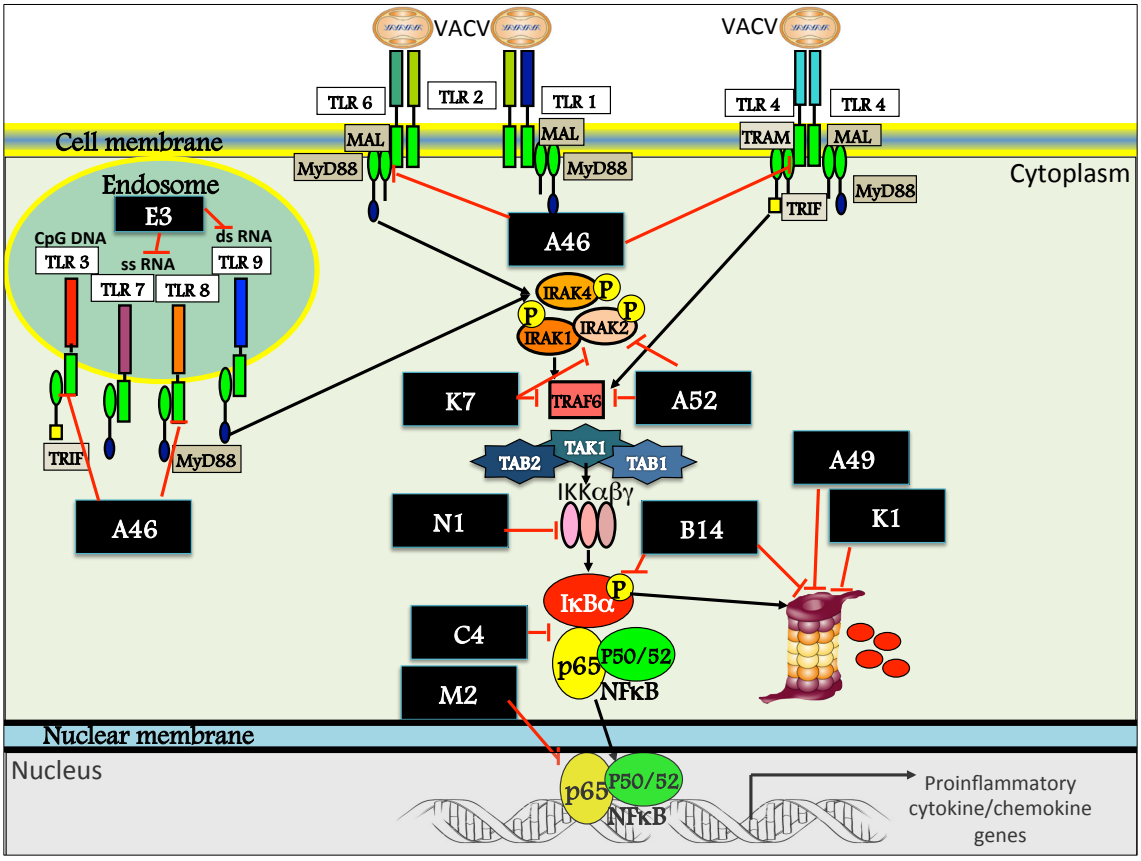

Figure 4. NFκB pathway and VACV inhibitors

(adapted from Smith et al., *J Gen Virol*, 2013)

1.2.3 Neutrophils

NFκB regulates the transcription of cytokine and chemokine pro-inflammatory signals for the recruitment of several cell types that act as the first cell line of defense in the innate immune response (Medzhitov, 2007), including monocytes, macrophages, dendritic cells (DC), natural killers (NK), B1 cells and granulocytes (Luster, 2002). Among the granulocytes, neutrophils are short-lived effector cells of the innate immune system that represent the first barrier against microbial pathogens (Mantovani et al., 2011). During microbial invasion, neutrophils (Figure 5) counteract infection immediately in a process termed microbial sterilization (Nathan, 2006), for which they engulf extracellular pathogens, infected and apoptotic cells (Hashimoto et al., 2007; Sorensen et al., 2001), they produce reactive oxygen intermediates (Borregaard, 2010), they release lytic enzymes and antimicrobial peptides from granules (Nathan, 2006), and they generate neutrophil extracellular traps (NET) (Brinkmann et al., 2004).

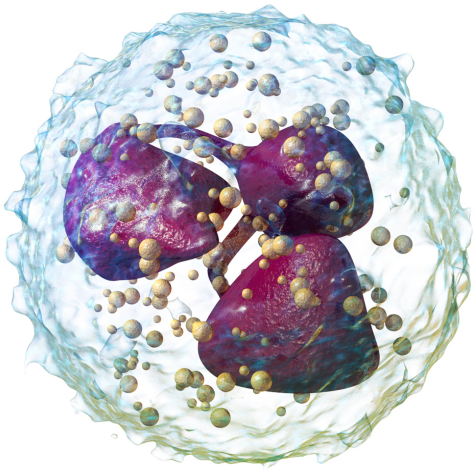

Figure 5. Neutrophil structure.
In purple the segmented nucleus and in yellow the granules (Blausen.com staff).

Recent studies demonstrate that neutrophils can also induce antigen-specific T cell responses by migrating to the lymph nodes (Chtanova et al., 2008) where they can efficiently cross-prime T cells (Beauvillain et al., 2007). Neutrophils polarize to distinct phenotypes in response to environmental signals (Araki et al., 2004). In the presence of granulocyte macrophage-colony-stimulating factor (GM-CSF) (Matsushima et al., 2013), TNF-α, IL-4 (Oehler et al., 1998) or IFN-γ (Fanger et al., 1997), neutrophils can adopt an antigen-presenting cell (APC) phenotype, thus triggering T cell activation. Neutrophils can acquire a macrophage (Abdel-Salam and Ebaid, 2008; Araki et al., 2004) or dendritic phenotype (Matsushima et al., 2013), and such hybrid neutrophil populations with APC-like properties participate in adaptive immune responses (Matsushima et al., 2013). The tumor cytokine/chemokine environment polarizes neutrophils; in the presence of TGF-β, neutrophil ability to activate CD8 T cells is impaired (Fridlender et al., 2009). In the tumor context, neutrophils can have intense phagocytic activity and induce T cell responses (Eruslanov

et al., 2014) or downregulate these functions and develop pro-tumorigenic properties (Mishalian et al., 2013).

During VACV infection, neutrophil infiltration is tissue-protective (Fischer et al., 2011), neutrophil recruitment to liver microvasculature with NET release defends the host cell from viral infection (Jenne et al., 2013), and activated neutrophils mediate antitumor effects by blocking vital blood supply to tumors (John et al., 2012). After intradermal and intranasal infection, MVA induces neutrophil recruitment (Abadie et al., 2009; Lehmann et al., 2009), which depends on complement component C5 and is mediated by CCR1 (chemokine receptor 1) (Price et al., 2015; Price et al., 2014).

It was recently shown that neutrophils generate polyclonal VACV-specific memory CD8 and not CD4 T cells. Via CCR1, the MVA-GFP^{+} neutrophils migrated directly from dermis to bone marrow without passing through draining lymph nodes (dLN). After intradermal MVA infection, neutrophils cannot prime CD8 T cells in the absence of myeloid APC in bone marrow (Figure 6), which suggests that neutrophils transport the antigens and then are engulfed by typical APC (Duffy et al., 2012).

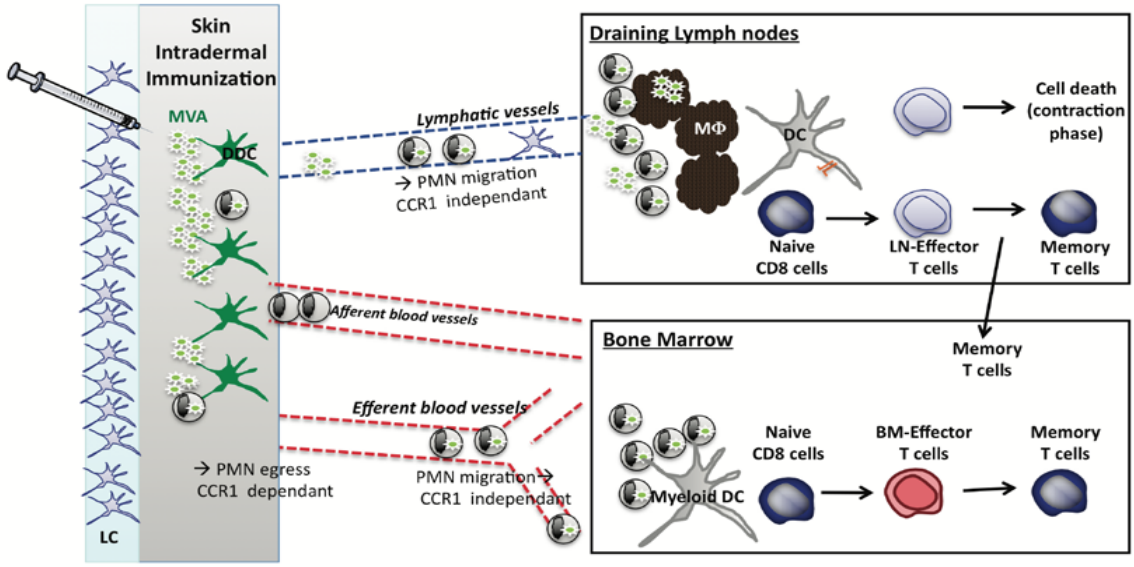

Figure 6. Scheme of neutrophil antigen transport

After intradermal injection of MVA, neutrophils transport the virus from the dermis to the bone marrow. Distinct phagocytic resident APC are involved in CD8 T cell priming. DC: dendritic cells, PMN: polymorphonuclear neutrophils, Mϕ: macrophages (from Duffy et al., *Immunity*, 2012).

1.3. T cell response

Antigen-specific T cell responses control and clear pathogen infections (Ha et al., 2008); for this reason, inducing T cells responses specific for the pathogen is one of the most important goal in vaccine effectiveness. The quality of this response, defined as T cell capacity to produce more than one cytokine after activation, is crucial for challenge protection (Seder et al., 2008). CD8 T cells protect against distinct parasites such as *Plasmodium falciparum* or *Trypanosoma cruzi*, the causative agents of malaria and Chagas disease, respectively (Reyes-Sandoval et al., 2011; Rigato et al., 2011), whereas CD4 T cells protect against malaria (Reece et al., 2004), influenza (Roman et al., 2002) and leishmaniasis (Darrah et al., 2007).

1.3.1 HIV: VACV vaccine and T cell response

HIV vaccine represents the best long-term solution to eradicate the worldwide HIV pandemic of 34 million infected people (Figure 7). The first HIV-envelope-based vaccines induced neutralizing antibodies that failed to protect against the virus due to he genetic variability of envelope proteins, which allows viral escape (Burton et al., 2004). Since most human HIV non-progressors preferentially maintain highly functional HIV-specific CD8 T cells (Betts et al., 2006), efforts focused on developing "T cell" vaccine candidates that control HIV replication, leading to attenuation of HIV disease and preventing secondary transmission (McMichael, 2006). Given the limited effectiveness of the ALVAC poxvirus vector in the RV144 phase III HIV/AIDS clinical trial (Rerks-Ngarm et al., 2009), there remains a need to improve poxvirus vector capacity as immunogen to increase protection levels; several strategies have been developed to improve T cell immunogenicity of HIV antigens (Gomez et al., 2012a). These approaches have yielded promising results in primates, and elicit protection after challenge with simian immunodeficiency virus (SIV) (Barouch et al., 2001; Barouch et al., 2013); in this infection model, CD4 and CD8 are associated with protection (Hansen et al., 2009).

In mice, recombinant MVA virus expressing envelope (Env) monomeric gp120 and the codon-optimized polyprotein Gag-Pol-Nef (GPN) from HIV-1 clade B, induced a strong, broad, polyfunctional and durable HIV-specific CD4 and CD8 T cell immune responses (Garcia-Arriaza et al., 2010; Garcia-Arriaza et al., 2011). In macaques, a similar MVA construct that expressed SIV Env and GPN antigens elicited a strong CD4

and CD8 T cell response that protected after SIV challenge (Mooij et al., 2008). Based on these results, this MVA-B vector entered a phase I clinical trial that demonstrated its safety and highly immunogenic profile (Cebere et al., 2006; Garcia et al., 2011; Mwau et al., 2004). In healthy human volunteers, MVA induced a robust and polyfunctional effector memory CD4 and CD8 T cell response to Gag and Env HIV antigens (Goonetilleke et al., 2006; Kibler et al., 2011).

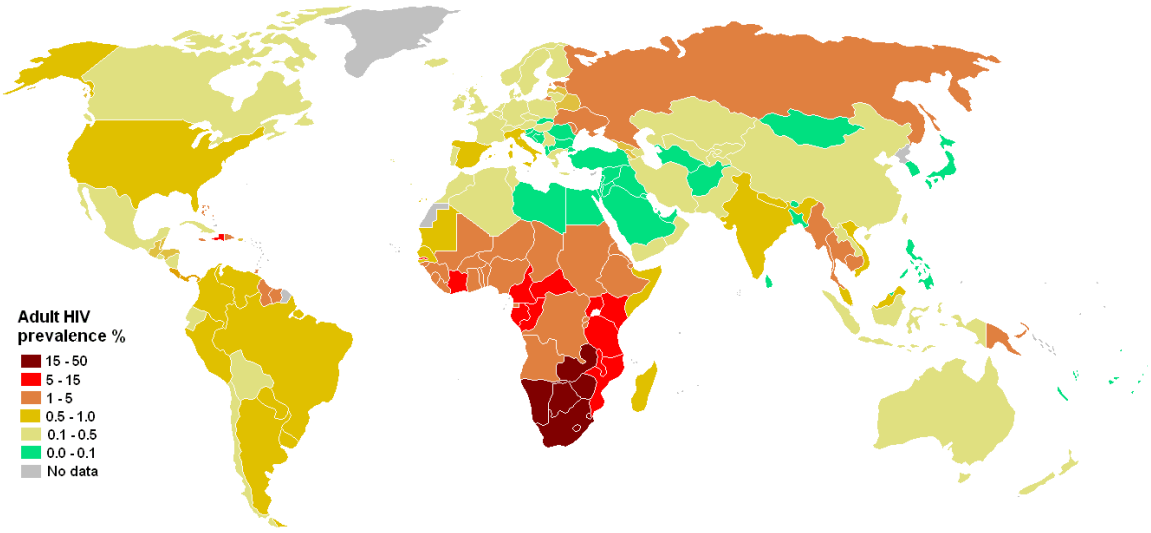

Figure 7. Estimated HIV prevalence in young adults (15-49 years) by country (www.unaids.org).

In mice, NYVAC induces a strong T cell response, with immunogenicity levels similar to those of MVA (Gomez et al., 2007b; Gomez et al., 2007c). Studies in non-human primates demonstrated that NYVAC expressing Env and/or GPN clade C HIV antigens elicited a balanced CD4/CD8 T cell response (Mooij et al., 2015) and robust T cell immunity when the virus was used to boost after priming with HIV Gag associated to DC-specific antibody (Flynn et al., 2011). Clinical trials showed the high NYVAC immunogenic potential to induce expansion of preexisting T cell responses as well as the appearance of newly detected polyfunctional CD4 and CD8 T cell responses in healthy volunteers and in chronically HIV-infected patients (Harari et al., 2008; Harari et al., 2012). Recent phase Ib clinical trials confirmed the ability of NYVAC to enhance HIV-specific T cell responses when this virus was used to boost after priming with recombinant adenovirus HIV-vector (Bart et al., 2014).

New HIV-NYVAC and MVA recombinants, with viral genes deleted, were tested in mice and demonstrated the ability to enhance the CD4 and CD8 T cell responses elicited by the parental MVA-B and NYVAC-C vectors (Garcia-Arriaza et al., 2014;

Gomez et al., 2012b). In this new generation of HIV vaccine candidates, MVA-C lacking the *C6L* gene alone (Garcia-Arriaza et al., 2011) or bearing the *K7R* (Garcia-Arriaza et al., 2013) or *A41L*/*B16R* genes (Garcia-Arriaza et al., 2010) specifically increase the Gag-Pol CD8 T cell response. In contrast, NYVAC-C that lacks the *B8R* gene alone or bears *B19R* (Gomez et al., 2012b), or MVA-B lacking the *N2L* gene (Garcia-Arriaza et al., 2014) specifically increased the Env CD8 T cell response.

1.3.2 Leishmania: VACV vaccine and T cell response

In human and in murine models of *Leishmania major* infection, polyfunctional CD4 T cells correlate with vaccine-mediated protection (Macedo et al., 2012). VACV that expresses LACK antigen protects partially against *Leishmania* infection (Gonzalo et al., 2001). MVA has been used as a safe, efficient vector for vaccination against leishmaniasis in mice (Perez-Jimenez et al., 2006), where it can induce a polyfunctional, long-term, LACK-specific CD4 T cell response (Sanchez-Sampedro et al., 2012). It was recently shown that M65, an attenuated, replication-competent vaccinia virus strain that expresses LACK antigen, preferentially induces a CD4 T cell response that correlates with protection after *Leishamina major* challenge (Sanchez-Sampedro et al., 2013).

Following *Leishmania major* infection, the T cell response is principally formed by T helper (Th) 2 cells, due to the Th2 immunogenicity of the LACK antigen (Mougneau et al., 1995). The Th2 response is associated to greater susceptibility to *Leishmania* infection in mice (Launois et al., 2007). Th1 cell lines expressing IL-2 and IFN-γ induced resistance to parasite infection compared to the Th2 clones expressing IL-4 and IL-5, which showed greater susceptibility (Scott et al., 1988). IL-4, -13 and -10 and TGF-β are the principal cytokines in the Th2 response and inhibit the macrophage activation that controls infection (Reiner and Locksley, 1995). IFN-γ and/or TNF-α (Th1 cytokines) regulate macrophage activation and, by inducing nitric oxide, control parasite expression (Figure 8) (Ding et al., 1988).

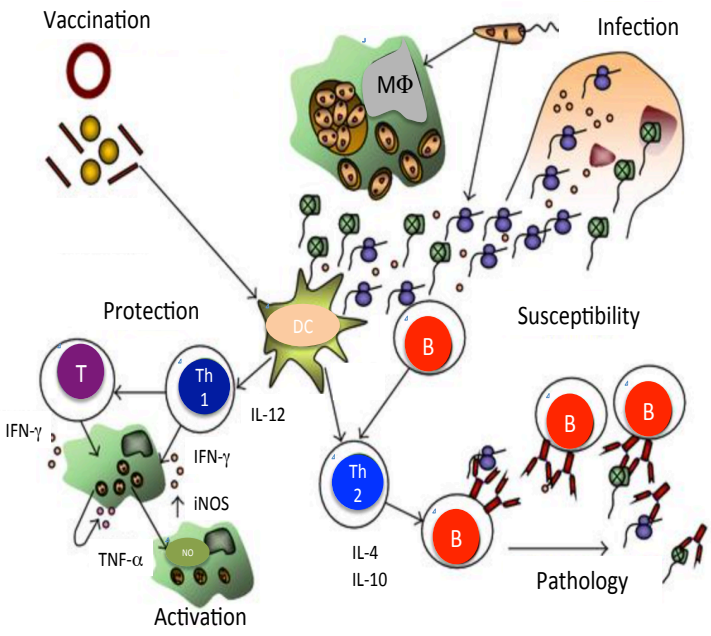

Figure 8. A model of pathology and protective capacity for *Leishmania* intracellular antigens. Intracellular parasite antigens are presented to the immune system during the natural course of infection and stimulate antigen-specific Th2-mediated humoral responses. The induction of a Th1 immune response is an approach to the development of *Leishmania* vaccines (adapted from Soto et al., *Scholarly Research Exchange*, 2009).

Although CD8 effector memory T cells are the major subtype present after human cutaneous leishmaniasis infection (Khamesipour et al., 2012) and probably induce Th1 polarization, CD8 T cells positive for CD107a (a surrogate marker for induction of cell killing) correlate with necrosis intensity and lesion size in leishmaniasis (Santos Cda et al., 2013). The magnitude of the CD4 T cell response is the major determining factor for the outcome of *Leishmania* infection (Xin et al., 2011), and CD4 T effector memory cells are necessary for protection against reinfection (Peters et al., 2014). IL-10 influences the magnitude and quality of the Th1 response, which correlates with protection against *Leishmania* infection in human (Darrah et al., 2010; Darrah et al., 2007).

1.4. Gene expression optimization

Optimization of poxvirus-based vaccines focuses on improving the generation of immune responses to the heterologous antigen. The regulation of antigen expression levels is an alternative vaccine design strategy adopted to induce antigen-specific immune responses (Wyatt et al., 2008).

The late-early vaccinia p7.5 promoter (Cochran et al., 1985) was the first used to induce heterologous antigen expression. Removal of poxvirus transcription termination signals from inserted genes (Earl et al., 1990), the regulation of gene expression under the bacteriophage T7 promoter (Fuerst et al., 1987), the vaccinia modified H5 (mH5) promoter (Wyatt et al., 1996) and the vaccinia short early-late synthetic promoter (pS) (Chakrabarti et al., 1997) have all been used as alternatives to p7.5 to increase the quantity of heterologous antigen expressed during infection.

1.4.1 VACV promoters

Studies of VACV show that the efficiency with which an antigen is processed and presented on the surface of infected cells influences its recognition by immune system cells (Moutaftsi et al., 2009); 90% of all VACV antigens recognized by CD8 T cells rank in the top 50% in terms of temporal mRNA expression (Sette et al., 2009). The timing of viral antigen expression correlates with the generation of antigen-specific CD8 T cell immune responses (Bronte et al., 1997). CD8 T cells specific to early expressed antigens have a proliferative advantage over late antigen-specific T cells (Kastenmuller et al., 2007). For this reason, efforts to develop new poxvirus vaccine candidates focus on using promoters to improve the timing of antigen expression and thus increase immune responses.

Vaccinia virus genes are classified as early, intermediate and late by the timing of expression after virus entry into the cell; their promoters have also been defined in these three classes (Yang et al., 2013; Yang et al., 2011). After the analysis of the VACV transcriptome, two research groups defined two categories of early genes based on their temporal expression (Assarsson et al., 2008; Yang et al., 2010a). Using a genome tiling array approach, Assarsson *et al*. differentiated the immediate-early genes from the early genes. Yang *et al*. used deep RNA sequence analysis to differentiate E1.1 from E1.2 genes as subclusters of early genes, and also defined a 15-nucleotide consensus sequence (AAAA-TGAAAA---A) that corresponds to the core of early gene promoters.

The core promoter of E1.1 genes corresponds more closely to the consensus sequence than those of E1.2 genes, suggesting that this difference could explain the readiness of E1.1 genes to be recognized by the transcription machinery compared to E1.2 genes (Yang et al., 2010b).

Based on these studies, endogenous poxviral early promoters have been compared with the p7.5 and pS promoters. The pC11R and pF11L early promoters induced high early antigens expression and T cell immunogenicity to the antigen; these levels were similar to those of p7.5 and pS promoters (Orubu et al., 2012). More recent studies demonstrated the possibility of designing poxvirus promoters to improve early antigen expression and antigen-specific T cell responses. Synthetic early promoters such as psFJ1-10 (Isshiki et al., 2014) or pHyb (Baur et al., 2010) and native early promoters like PrMVA13.5-long (Wennier et al., 2013) have early repeated core sequences. Compared to p7.5 and pS, these new promoters, which differ in their early motif sequence and in spacer lengths between the gene and the core promoter, are able to increase expression of heterologous antigens and the antigen-specific immune responses. They are prototypes for the generation of safe recombinant poxvirus-based vaccines to potentiate antigen expression and the immune responses.

OBJECTIVES

2. Objectives

Given the current interest in vaccinia virus (VACV) as vaccine vector and its optimization as immunogen, various strategies and distinct VACV-based vaccine candidates have been developed to improve foreign antigen-specific T cell immune responses.
This work is focused on:

- VACV-dependent activation of the NFκB signaling pathway to increase neutrophil migration and T cell responses to HIV antigens

- VACV promoter modifications to enhance antigen expression and T cell responses to GFP and *Leishmania* LACK antigens.

We proposed the following objectives:

1. Define the mode of action of VACV inhibitors on the NFκB pathway
2. Describe how VACV modulates the innate immune response
3. Analyze the role of neutrophils in the generation of T cell responses
4. Characterize HIV-specific T cell responses
5. Identify new VACV early promoters
6. Understand the role of the early promoter sequence in early antigen expression
7. Demonstrate the role of early promoter spacers in early antigen expression
8. Study how the timing of antigen expression influences T cell responses
9. Examine the GFP-specific T cell response profile
10. Characterize the *Leishmania* LACK-specific T cell responses.

RESULTS

3.1 Summary

3.1.1 NFκB activation by modified vaccinia virus as a novel strategy to enhance neutrophil migration and HIV-specific T cell responses

In the vaccine research, there is major interest in understanding poxvirus vector function in the host and its manipulation to trigger optimal immune responses to HIV antigens. Whereas attenuated vaccinia virus (VACV) is a commonly used vector model for vaccine development, poxviruses use a sophisticated strategy to escape immune surveillance by expressing inhibitory molecules that could compromise immune response and thus, vaccine efficiency. In the context of VACV infection, neutrophils generate virus-specific CD8 T cells, but it was nonetheless not known how VACV modulates neutrophil recruitment and its significance in immune response triggering.

To respond to these questions, we generated NYVAC, a highly attenuated VACV strain, that expresses HIV-1 clade C antigens but lacks specific viral genes (*A52R*, *K7R*, *B15R*). These genes encode NFκB pathway inhibitors that limit pro-inflammatory cytokine/chemokine-dependent neutrophil recruitment. Using single, double and triple deletion mutants, we demonstrated that these genes cooperate to inhibit the NFκB signaling pathway, and that combined deletion was necessary for efficient pathway triggering.

Mice treated with this NYVAC-C ΔA52RΔB15RΔK7R vector showed enhanced immune responses, based on increased neutrophil recruitment to the viral infection site. This recruitment depended on NFκB pathway activation and was essential for induction of T cell immune responses to the HIV Gag/Pol antigens delivered by the vector. To generate this response, outgoing neutrophil traffic from the infection site to various lymphoid organs was necessary. After virus infection, NFκB pathway activation led to expression of several cytokines and chemokines that recruit specific neutrophil populations (Nα and Nβ) to the infection site. Nβ neutrophils had an enhanced activation profile, higher levels of antigen-presenting cell markers, and greater capacity to induce antigen-specific T cell activation than Nα cells. These findings suggest a design strategy for poxvirus vector use as vaccines.

NFκB activation by modified vaccinia virus as a novel strategy to enhance neutrophil migration and HIV-specific T-cell responses

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Neutrophils are antigen-transporting cells that generate vaccinia virus (VACV)-specific T-cell responses, yet how VACV modulates neutrophil recruitment and its significance in the immune response are unknown. We generated an attenuated VACV strain that expresses HIV-1 clade C antigens but lacks three specific viral genes (*A52R*, *K7R*, and *B15R*). We found that these genes act together to inhibit the NFκB signaling pathway. Triple ablation in modified virus restored NFκB function in macrophages. After virus in-fection of mice, NFκB pathway activation led to expression of several cytokines/chemokines that increased the migration of neutrophil populations (Nα and Nβ) to the infection site. Nβ cells displayed features of antigen-presenting cells and activated virus-specific CD8 T cells. Enhanced neutrophil trafficking to the infection site correlated with an increased T-cell response to HIV vector-delivered antigens. These results identify a mechanism for poxvirus-induced immune response and alternatives for vaccine vector design.

vaccinia virus | neutrophils | NFκB | vaccine | HIV

Although attenuated vaccinia virus (VACV) is a commonly used vector model for vaccine development (1), poxviruses have a sophisticated strategy to escape immune surveillance by expressing inhibitory molecules, which could compromise immune response and thus vaccine efficiency. VACV encodes several proteins involved in host immune evasion that limit virus recognition by innate immune cells such as neutrophils and could affect the virus's ability to induce adaptive immunity (2).

During microbial invasion, neutrophils counteract infection immediately in a process termed microbial sterilization (3), for which they engulf extracellular pathogens and infected and apoptotic cells (4, 5), they produce reactive oxygen intermediates (6), they release lytic enzymes and antimicrobial peptides from their granules (3), and they generate neutrophil extracellular traps (7). Neutrophils can induce an adaptive immune response after migrating to the lymph nodes (8) and efficiently cross-prime naïve T cells (9). In the presence of a granulocyte macrophage–colony-stimulating factor (GM-CSF) (10), tumor necrosis factor (TNF)-α, interleukin (IL)-4 (11), or interferon (IFN)-γ (12), neutrophils can adopt an antigen-presenting cell (APC) phenotype, thus triggering T-cell activation. In the context of VACV infection, neutrophils generate virus-specific memory CD8 T cells, transporting antigens from the dermis to the bone marrow (BM) (13). It is nonetheless not known how VACV modulates neutrophil recruitment, the factors involved, or the significance in immune response triggering.

Proinflammatory cytokine/chemokine production by an infected cell, which signals the recruitment of neutrophils involved in the immune response, is limited by inhibition of signaling pathways such as nuclear factor kappa B (NFκB) (2). NFκB pathway activation depends on the virus's capacity to interact with Toll-like receptors (TLRs). Viral pathogen-associated molecular patterns interact with TLR2 and TLR4 on the plasma membrane (14). Pathogen-associated molecular pattern binding induces TLR2 and TLR4 homo- or heterodimerization (15), followed by recruitment of Toll/IL-1 receptor (TIR) domain-containing adaptor proteins such as myeloid differentiation factor 88 (MyD88) and TIR domain-containing adapter protein (TIRAP)/MyD88 adaptor-like (MAL) or TIR domain-containing adapter-inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) (14). In the MyD88-dependent and the TRIF-dependent pathways, activation of TNF receptor-associated factor 6 results in IκBα phosphorylation and degradation, allowing release of the NFκB complex, composed of p65/p50 or p65/p52 heterodimers, into the cell nucleus. NFκB then binds the κB site and regulates transcription of proinflammatory cytokine and chemokine genes (14).

VACV interacts with TLR2 (16) and TLR4 (17) and produces early direct or indirect viral inhibitors of the NFκB pathway such as A46 (18), A49 (19), A52 (18), B14 (20), C4 (21), E3 (22), K1 (23), K7 (24), M2 (25), and N1 (26). New York vaccinia virus (NYVAC), a highly attenuated VACV strain used as vaccine vector, lacks most of these proteins but encodes NFκB pathway

Significance

Although poxvirus vectors are widely used in preclinical and clinical trials as candidate vaccines for multiple pathogens, how these vectors affect the host immune response is not clear. In this study, we developed a poxvirus vector based on the attenuated New York vaccinia virus (NYVAC), which is able to target a central host-cell signaling pathway, NFκB. In mice, the modified NYVAC acts on the immune system by increasing specific neutrophil migration via NFκB activation and in turn enhances CD8 T-cell responses to HIV antigens delivered by the viral vector. We show that these inherent properties define a mechanism for poxvirus-induced immune responses and offer novel approaches to vaccine vector design.

Author contributions: M.E. supervised the work; M.D.P., E.M.-P., M.Z., M.T., L.P., and M.E. designed research; M.D.P., E.M.-P., and M.Z. performed research; M.D.P., E.M.-P., B.P., C.E.G., J.N., J.L.N., C.C., and G.P. contributed new reagents/analytic tools; M.D.P., E.M.-P., and C.O.S.S. analyzed data; and M.D.P. wrote the paper.

The authors declare no conflict of interest.

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inhibitors A52, K7, and B15 (corresponding to B14 in the Western Reserve strain) (2).

Given the interest in attenuated VACV vectors as vaccine candidates and their optimization as immunogens (1), we generated several NYVAC deletion mutants that express HIV-1 Envelope (Env) and group-specific antigen (Gag)–polymerase (Pol)–negative regulatory factor (Nef) clade C antigens (NYVAC-C) but lack specific genes to encode NFκB inhibitors. We show that A52, K7, and B15 viral proteins inhibit the NFκB pathway in macrophages and that infection with a virus that lacks these three inhibitors results in NFκB pathway activation. NFκB-dependent induction of cytokines and chemokines is enhanced in vitro and in vivo by NYVAC-C ΔA52RΔB15RΔK7R and is essential for specific neutrophil recruitment to the infection site. Neutrophils acquire features of APCs, activate antigen-specific T cells, and migrate to the draining lymph nodes and to the spleen, where they induce antigen-specific CD8 T-cell responses. These findings provide important insights into the mechanism of the poxvirus-induced immune response, which is relevant in vaccine vector design.

Results

Concomitant Deletion of *A52R*, *B15R*, and *K7R* Genes in NYVAC Leads to Enhanced NFκB Pathway Activation. To define the immune modulatory role of the VACV viral genes that antagonize the NFκB pathway, we generated single, double, and triple deletion mutants for the viral genes encoding A52, K7, and B15 (*Materials and Methods*), using as a backbone the NYVAC-C vector expressing HIV-1 clade C antigens Env (gp120) as a cell-released product and Gag-Pol-Nef (GPN) as an intracellular polyprotein (27). With this targeted gene disruption, we generated the following NYVAC deletion mutants: NYVAC-C ΔB15R, NYVAC-C ΔA52R, NYVAC-C ΔK7R, NYVAC-C ΔB15RΔK7R, NYVAC-C ΔA52RΔB15R, NYVAC-C ΔA52RΔK7R, and NYVAC-C ΔA52RΔB15RΔK7R (also termed triple deletion mutant or NYVAC-C Δ3 for short). Gene deletion was confirmed by PCR, using primers annealed to flanking sequences of each gene (Fig. S1*A*). The gp120 and GPN HIV-1 proteins were expressed correctly by each deletion mutant, as confirmed by Western blot (Fig. S1*B*).

To determine the contribution of the NYVAC inhibitors to NFκB activation, we infected murine J774 macrophages with the different single, double, and triple NYVAC-C deletion mutants and performed a time-course assay to evaluate IκBα phosphorylation. We observed a clear increase in the level of IκBα phosphorylation in NYVAC-C Δ3-infected J774 cells compared with the parental or the other deletion mutants. As predicted, this phosphorylation was accompanied by enhanced IκBα degradation (Fig. 1*A*). Similar results between parental virus and NYVAC-C Δ3 were obtained following infection of human THP-1 monocytes differentiated into macrophages (Fig. S2*A*).

To confirm that increased IκBα phosphorylation in triple deletion mutant-infected cells enhanced NFκB activity, we used electrophoretic mobility shift assay (EMSA) to analyze NFκB binding to its consensus binding sequence motif and an immunofluorescence assay to detect p65 translocation from the cytoplasm to the nucleus. EMSA indicated marked NFκB pathway activation in NYVAC-C Δ3-infected macrophages, with a two-fold increase from 3 to 5 h postinfection compared with NYVAC-C–infected macrophages (Fig. 1*B*), which was confirmed by immunofluorescence of p65 migration from the cytoplasm to the nucleus (Fig. 1*C*). These results suggest that these genes (*A52R*, *K7R*, and *B15R*) must be removed concomitantly from the viral vector to induce robust pathway activation following VACV infection.

A variety of cytokines and chemokines are known NFκB target genes. We therefore analyzed their secretion in supernatants of J774 macrophages infected with the various deletion mutants. Compared with parental and other deletion mutant viruses, only NYVAC-C Δ3-infected cells showed significantly increased

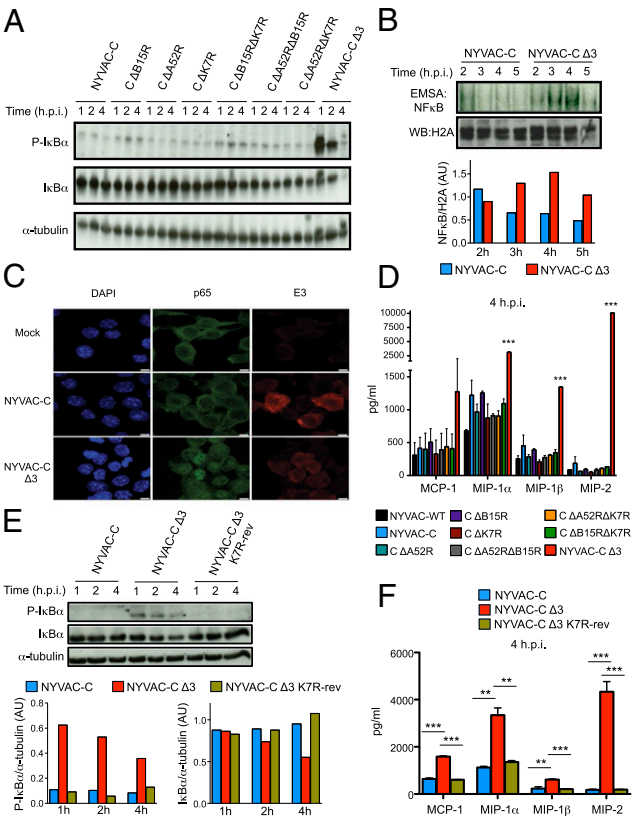

Fig. 1. Deletion of *A52R*, *B15R*, and *K7R* genes induces robust NFκB activation. (*A*) Phosphorylated and total IκBα forms analyzed by Western blot (WB) in J774 mouse macrophages infected with NYVAC-C or NYVAC-C deletion mutants (5 PFUs per cell) for 1, 2, and 4 h. α-tubulin was used as the internal loading control. (*B*) J774 nuclear extracts incubated with ^{32}P-end–labeled NFκB probe and assayed by EMSA for DNA binding complexes at 2, 3, 4, and 5 h postinfection with NYVAC-C or NYVAC-C Δ3. Histone H2A was used as the internal loading control for WB. Bars show the ratio of NFκB probe bands to histone H2A bands, quantified using ImageJ. (*C*) Confocal microscopy of mock-, NYVAC-C–, or NYVAC-C Δ3-infected J774 cells (3 h postinfection). Cells were stained with antibodies to E3 VACV protein (red), p65 cell protein (green), and DAPI probe to detect DNA (blue). (Scale bars, 5 μm.) (*D*) Concentrations of chemokines MCP-1, MIP-1α, MIP-1β, and MIP-2 at 4 h postinfection, quantified by immunoassay in supernatants of J774 cells infected as indicated. (*E*) Phosphorylated and total IκBα forms analyzed by WB in J774 mouse macrophages infected with NYVAC-C, NYVAC-C Δ3, or NYVAC-C Δ3 K7R-rev (5 PFUs per cell) for 1, 2, and 4 h. α-tubulin was used as the internal loading control. Bars show the ratio of phospho- or total IκBα bands to α-tubulin bands, quantified with ImageJ. (*F*) Concentrations of chemokines MCP-1, MIP-1α, MIP-1β, and MIP-2 at 4 h postinfection, quantified by immunoassay in supernatants of NYVAC-C–, NYVAC-C Δ3-, or NYVAC-C Δ3 K7R-rev–infected J774 macrophages. Values show mean ± SEM of duplicates, representative of three independent experiments. ***$P < 0.001$, **$P < 0.01$.

production of cytokines such as TNF-α and IL-6 at 24 h postinfection (Fig. S2*B*) and of chemokines such as macrophage inflammatory proteins (MIPs)-1α, -1β, and -2 at 4 h postinfection (Fig. 1*D*). We observed a similar chemokine/cytokine profile in primary peritoneal macrophages infected with the NYVAC-C deletion mutants (Fig. S2*C*). These data indicate enhanced NFκB-dependent chemokine and cytokine secretion after macrophage infection with the NYVAC-C Δ3.

To demonstrate that only one of the three inhibitory molecules is sufficient to abolish NFκB activation, we generated the revertant NYVAC-C Δ3 K7R-rev virus, in which the *K7R* gene was reinserted in the HA locus (Fig. S1*C*); expression was confirmed by RT-PCR (Fig. S1*D*). IκBα phosphorylation, degradation, and chemokine secretion levels in NYVAC-C Δ3 K7R-rev–infected

J774 macrophages resembled those of the same cells infected with NYVAC-C (Fig. 1 *E* and *F*).

Increased in Vitro and in Vivo Neutrophil Migration After NYVAC-C Δ3 Infection. Because chemokines such as MCP-1, MIP-1α, -1β, and -2 or keratinocyte chemoattractant (KC) are essential for recruitment of several cell types, we tested whether a specific cell subset responds to this chemokine–cytokine mixture. We used an in vitro migration assay in which total murine BM cells were allowed to migrate after stimulation with supernatants of the different NYVAC-infected (4 h) J774 macrophages. Only neutrophils ($Ly6G^+CD11b^+$) exposed to NYVAC-C Δ3-infected macrophage supernatants showed significantly more migration compared with the parental virus or the other deletion mutants (Fig. 2*A*). Migrated neutrophils showed the same percentages of early and late apoptotic cells when stimulated with supernatants of NYVAC-C– and NYVAC-C Δ3-infected J774 macrophages (Fig. S3*A*).

In a similar experiment, we used a supernatant of J774 cells pretreated with JSH-23, an inhibitor of p65 translocation to the nucleus (28), and then infected it with the NYVAC virus. The number of neutrophils that migrated toward supernatants of JSH-23–treated NYVAC-C Δ3-infected cells was significantly lower than those that migrated to supernatants of untreated NYVAC-C Δ3-infected cells and was similar to the other NYVAC-C deletion mutants (Fig. 2*A*). These data show that NFκB activation underlies the differences in neutrophil migration induced by the triple deletion mutant virus and its parental strain.

To determine whether infection with the triple mutant virus also leads to increased neutrophil migration in vivo, BALB/c mice were infected by i.p. injection of 10^7 plaque-forming units (PFUs) of NYVAC-C or NYVAC-C Δ3. Peritoneal exudate cells (PECs) were collected 1 h postinfection to study NFκB activation. In PECs of NYVAC-C Δ3-infected mice, IκBα phosphorylation was significantly higher than in PECs of parental virus-infected mice (Fig. 2*B*). At 3 and 6 h postinfection, mice that received the triple deletion mutant virus showed a significant increase in neutrophils ($Ly6G^+CD11b^+$) in the peritoneal cavity compared with NYVAC-C–infected mice (Fig. 2 *C* and *D*). There were no differences in monocytes (M), macrophages (Mϕ), B cells (B), or T cells (T) (Fig. 2 *C* and *D*), which indicated that in the first 6 h of infection, the triple deletion mutant virus promotes neutrophil migration to the infection site before migration of the other hematopoietic cells. After 12 h, we detected no difference in neutrophil migration, but triple deletion mutant virus-infected mice showed a significant increase in monocytes compared with NYVAC-C–infected mice; resident macrophages were absent for both viruses (Fig. 2*E*). The infection kinetics indicates that neutrophil migration precedes monocyte migration in NYVAC-C Δ3-infected mice.

To rule out the possibility that increased neutrophil recruitment was caused by differences in virus infective capacity, we replaced the HIV-1 antigen cassette with the GFP gene to generate NYVAC-GFP and NYVAC-GFP Δ3 viruses, which were injected (10^7 PFUs; i.p.) into BALB/c mice. At 3 h postinfection, comparison of NYVAC-GFP Δ3- and NYVAC GFP-injected mice showed similar percentages of GFP^+ cells in PEC types (neutrophils, monocytes, macrophages, and B and T cells; Fig. 2*F*). These percentages indicate that both viruses have similar in vivo infective capacities and that macrophages, which constitute ∼20% of the total peritoneal cell yield, are the most susceptible cell type to NYVAC infection (∼80%). The absolute numbers of GFP^+ cells indicated that only GFP^+ neutrophils were significantly higher in NYVAC-GFP Δ3- than in NYVAC-GFP–infected mice (Fig. S3*B*), due to a significant increase in neutrophil migration in NYVAC-GFP Δ3- compared with NYVAC-GFP–infected mice (Fig. S3*C*).

The peritoneal exudates of NYVAC-C Δ3-infected mice (6 h postinfection) showed significantly higher levels of cytokines

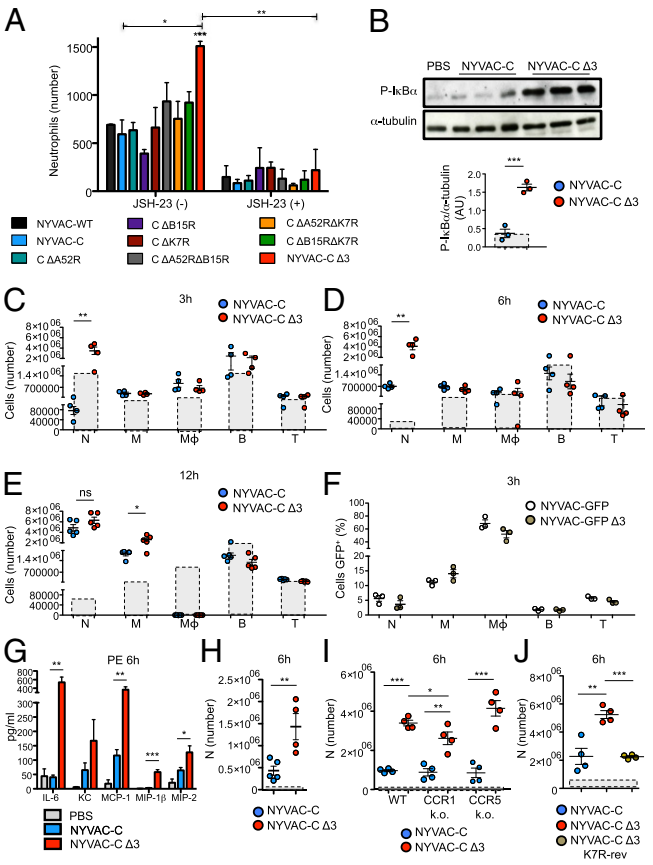

Fig. 2. Increased neutrophil recruitment after NYVAC-C Δ3 infection. (*A*) Absolute number of BM neutrophils that migrated after stimulation with supernatants of virus-infected J774 cells (4 h). J774 cells were untreated or pretreated with the p65 inhibitor JSH-23 (30 μM). (*B*) Phosphorylated IκBα form analyzed by WB in PECs of BALB/c mice at 1 h after injection with PBS or 10^7 PFUs of NYVAC-C or NYVAC-C Δ3. α-tubulin was used as the internal loading control. Each point represents the ratio of phospho-IκBα to α-tubulin bands in individual mice, as quantified by ImageJ. Boxes with dashed lines indicate the ratio for PBS-injected mice. Absolute numbers of neutrophils (N), monocytes (M), macrophages (Mϕ), B cells (B), and T cells (T) in the peritoneal cavity of BALB/c mice at 3 h (*C*), 6 h (*D*), and 12 h (*E*) after injection of 10^7 PFUs of NYVAC-C or NYVAC-C Δ3. Boxes with dashed lines indicate absolute numbers for PBS-injected mice. (*F*) Percentages of GFP^+ neutrophils (N), monocytes (M), macrophages (Mϕ), B (B), and T cells (T) in the peritoneal cavity of NYVAC-GFP– or NYVAC-GFP Δ3-injected (10^7 PFUs) mice at 3 h postinfection. (*G*) Cytokine/chemokine levels at 6 h postinfection in peritoneal exudates of PBS-, NYVAC-C–, or NYVAC-C Δ3- (10^7 PFUs) injected mice ($n = 5$ per group). (*H*) Absolute number of neutrophils (N) in the peritoneal cavity of mice 6 h after injection of NYVAC-C– or NYVAC-C Δ3-peritoneal exudates at 6 h postinfection. Boxes with dashed lines indicate total numbers in PBS-peritoneal exudate-injected mice. (*I*) Absolute numbers of neutrophils (N) in the peritoneal cavity of C57/BL6J WT, CCR1 KO, and CCR5 KO mice at 6 h after injection with PBS or 10^7 PFUs of NYVAC-C or NYVAC-C Δ3. Boxes with dashed lines indicate absolute numbers of cells in PBS-injected mice. (*J*) Absolute numbers of neutrophils (N) in the peritoneal cavity of BALB/c mice at 6 h after injection with 10^7 PFUs of NYVAC-C, NYVAC-C Δ3, or NYVAC-C Δ3 K7R-rev. Boxes with dashed lines indicate absolute numbers of cells in PBS-injected mice. Graphs show mean ± SEM; each point represents an individual mouse. Data are representative of two independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

such as IL-6 and chemokines such as MCP-1, MIP-1β, and MIP-2 compared with those of NYVAC-C–infected mice (Fig. 2*G*). We detected no difference in IL-8 secretion between these two groups (Fig. S3*D*). To demonstrate that the neutrophils were recruited by increased cytokine/chemokine secretion at the infection site, peritoneal exudate pools of 6-h NYVAC-C– and NYVAC-C Δ3-infected mice, free of PECs and viruses, were

injected i.p. into mice, and neutrophils in the peritoneal cavity were analyzed at 6 h postinfection. Total neutrophils in NYVAC-C Δ3-peritoneal exudate-injected mice were significantly higher compared with those in NYVAC-C–peritoneal exudate-injected mice (Fig. 2*H*). These data indicate that enhanced neutrophil recruitment was dependent on cytokine/chemokines produced during the infection.

To determine the chemokine(s) involved in increased neutrophil migration in NYVAC-C Δ3-infected mice, we performed the same experiment in C57/BL6J CCR1 and CCR5 knockout (KO) mice, which are deficient in the CCR1 and CCR5 receptors involved in binding the chemokines MIP and MCP. Total neutrophil number in the peritoneal cavity of NYVAC-C Δ3-infected CCR1 KO mice was significantly lower compared with NYVAC-C Δ3-infected wild-type (WT) mice, although not as low as in NYVAC-C–infected CCR1 KO mice (Fig. 2*I*). There were no differences between CCR5 KO and WT mice (Fig. 2*I*). These data suggest that the increased neutrophil migration is mediated partially by CCR1 and not by CCR5.

To demonstrate that the enhanced neutrophil migration in NYVAC-C Δ3-infected mice is NFκB-dependent, we infected mice with NYVAC-C Δ3 K7R-rev and analyzed PEC neutrophils at 6 h postinfection. The revertant virus induced a significant decrease in neutrophil number compared with the NYVAC-C Δ3 virus and resembled the value for the NYVAC-C virus (Fig. 2*I*).

Enhanced Neutrophil Trafficking After NYVAC-C Δ3 Infection. To determine how neutrophil trafficking after NYVAC-C Δ3 infection occurs, we quantified neutrophils in BM, spleen, lymph nodes, peripheral blood, and omentum. We included omentum in this analysis as it is the primary site of neutrophil exudation and mediates recruitment from circulation to the peritoneal cavity. NYVAC-C Δ3-infected mice had significantly fewer neutrophils in BM but more neutrophils in blood and a larger percentage of neutrophils in omentum than NYVAC-C–infected mice (Fig. 3*A*). These changes were the result of increased IL-6, KC, MCP-1, and MIP-1β production in sera of triple deletion mutant-infected mice compared with those infected with the parental strain (Fig. 3*B*). No differences were detected in IL-8 levels between the two groups (Fig. S3*E*). Compared with neutrophils, at 6 h NYVAC-C Δ3-infected mice had significantly more monocytes ($CD115^+$) in the blood than NYVAC-C–infected mice, but we found no difference between the two groups in the percentage of monocytes in BM or omentum (Fig. S3*F*).

In the secondary lymphoid organs such as the spleen and mediastinal lymph nodes (MLNs), we found significantly more neutrophils in NYVAC-C Δ3- than in NYVAC-C–infected mice (Fig. 3 *C* and *D*). In MLNs and in the spleen of NYVAC-C Δ3-infected mice, the absolute number of neutrophils was significantly reduced from 6 to 12 h postinfection (Fig. 3 *C* and *D*). We also detected a significant increase in neutrophil death from 6 to 12 h in the spleens of NYVAC-C Δ3- compared with NYVAC-C–infected mice (Fig. S3*G*), as well as a clear trend ($P = 0.07$) in increased MLN neutrophil death (Fig. S3*H*). This increase could explain the significant reduction in spleen and MLN neutrophils from 6 to 12 h.

To test a common human immunization route, BALB/c mice also received intramuscular injections of NYVAC-C or NYVAC-C Δ3 viruses. NYVAC-C Δ3 induced a significant neutrophil increase in the spleen at 12 h postinfection (Fig. 3*E*) and in draining popliteal lymph nodes (PLNs) at 6 and 12 h postinfection (Fig. 3*F*) compared with NYVAC-C.

These data indicate that neutrophil migration occurs in a specific time window and is enhanced after NYVAC-C Δ3 infection. Neutrophils abandon BM, enter the blood stream, and through the omentum reach the infection site, where they encounter infected cells; they subsequently leave toward secondary lymphoid organs to initiate an adaptive immune response.

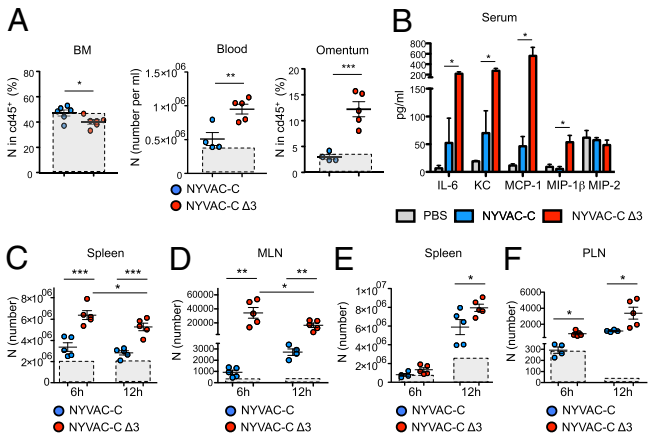

Fig. 3. Increased neutrophil trafficking after NYVAC-C Δ3 infection. (*A*) Percentages of neutrophils in BM and omentum, and total number of neutrophils in blood at 6 h postinfection, from NYVAC-C– or NYVAC-C Δ3-injected mice. Boxes with dashed lines indicate absolute numbers of cells in PBS-injected mice. (*B*) Cytokine/chemokine levels at 6 h postinfection in serum of PBS-, NYVAC-C–, or NYVAC-C Δ3-injected mice ($n = 5$ per group). Shown are total neutrophil numbers in spleen (*C*) and MLNs (*D*) at 6 and 12 h postinfection, from NYVAC-C– or NYVAC-C Δ3-injected mice, as well as the total neutrophil numbers in spleen (*E*) and PLNs (*F*) at 6 and 12 h post-intramuscular infection, from NYVAC-C– or NYVAC-C Δ3-injected mice. Boxes with dashed lines indicate absolute numbers of cells in PBS-injected mice. Graphs show mean ± SEM; each point represents an individual mouse. Data are representative of two independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Neutrophils Act as APCs After NYVAC-C Δ3 Infection. We next characterized the neutrophil population recruited to the peritoneum after infection. Using flow cytometry, we found two populations that differed in cell size and complexity, which we termed Nα and Nβ (Fig. 4*A*). To evaluate the morphology of Nα and Nβ populations, we sorted $Ly6G^+$ cells. Both populations had clear neutrophil-like morphology, but Nβ cells were larger, more lobulated, and more hypersegmented than Nα cells (Fig. 4*B*). In PECs, the Nα subset showed a fourfold increase, whereas the Nβ subset showed a 20-fold increase in NYVAC-C Δ3-infected compared with parental virus-infected mice (Fig. 4*C*).

We tested whether acquisition of the Nβ profile depended on direct virus infection of neutrophils or on the cytokine/chemokine milieu produced after infection. Mice were initially infected with NYVAC-C or NYVAC-C Δ3 to induce neutrophil recruitment to the peritoneal cavity and subsequently injected with NYVAC-GFP or NYVAC-GFP Δ3 to infect the migrated neutrophils. Most GFP^+ neutrophils in mice infected with the parental or triple deletion mutant virus had a Nβ-like profile (Fig. 4*D*), indicating that Nβs were more susceptible to the infection than Nα cells. The majority of Nβ neutrophils were GFP^- in both mouse groups (Fig. 4*E*), which indicated that neutrophil viral infection was not directly responsible for Nβ subset generation. As predicted, the Nβ population (GFP^+ and GFP^-) was higher in the NYVAC-C Δ3- than in NYVAC-C–infected mice (Fig. 4*E*). To demonstrate that Nβ and Nα were recruited by increased cytokine/chemokine secretion at the infection site, peritoneal exudate pools of 6 h NYVAC-C– and NYVAC-C Δ3-infected mice, free of PECs and viruses, were injected i.p. into mice; neutrophils in the peritoneal cavity were analyzed at 6 h postinfection. The absolute numbers of Nα and Nβ neutrophils in NYVAC-C Δ3-peritoneal exudate-injected mice were significantly higher than those of NYVAC-C–peritoneal exudate-injected mice (Fig. S4*A*).

Examination of Nβ neutrophils in the spleen and MLNs of NYVAC-C Δ3-infected mice showed significantly more Nβ cells than in NYVAC-C–infected mice (Fig. 4*F*).

To characterize Nα and Nβ neutrophil phenotype in NYVAC-C Δ3-infected mice, we analyzed the expression of several surface markers including the CD11b activation marker involved in neutrophil adhesion and migration, MHC class I and class II molecules used for presentation of antigenic determinants to T cells, the CD11c dendritic cell (DC) marker, and CD80/CD86 APC costimulatory markers involved in T-cell activation. All neutrophils expressed CD11b, MHC class I, and CD80 markers. The percentage of Nβ neutrophils that expressed MHC class II, CD11c, or CD86 was significantly higher than Nα cells (Fig. 4*G*). Moreover, levels of all surface markers were higher in Nβ than in Nα neutrophils, with significant differences in most cases (Fig. 4*H*). These results indicate that Nβs have a more APC-like profile than Nα neutrophils.

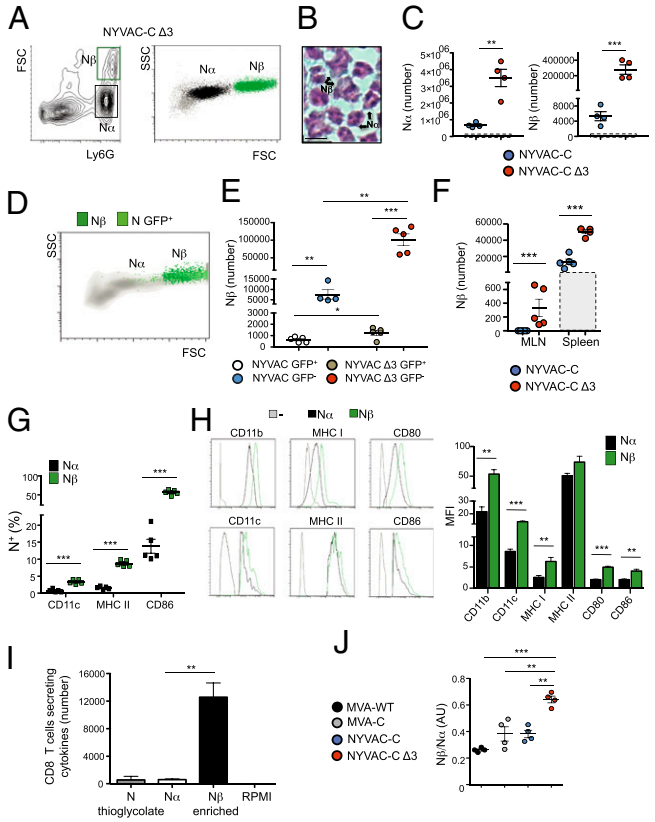

Fig. 4. Nβ neutrophil population with APC features is involved in specific activation of CD8 T cells after NYVAC-C Δ3 infection. (*A*) FACS plots determined by forward scatter (FSC) and Ly6G, and side scatter (SSC) and FSC of PEC from NYVAC-C Δ3-injected mice. Nα (black) and Nβ (green) neutrophil subsets are shown as dot plots. (*B*) Hematoxylin/eosin staining of peritoneal neutrophils presorted for Ly6G$^+$. (Scale bar, 10 μm.) (*C*) Absolute number of Nα and Nβ neutrophils in the peritoneal cavity at 6 h postinfection in NYVAC-C– or NYVAC-C Δ3- (10^7 PFUs) injected mice. Boxes with dashed lines indicate total number in PBS-injected mice. Graphs show mean ± SEM; each point represents an individual mouse. (*D*) Nβ (dark green) and infected neutrophils (N GFP$^+$; light green) from PECs of NYVAC-GFP Δ3-infected mice. (*E*) Absolute numbers of Nβ GFP$^{+/-}$ neutrophils in NYVAC-C (10^7 PFUs)/NYVAC-GFP– (10^7 PFUs) or NYVAC-C Δ3 (10^7 PFUs)/NYVAC-GFP Δ3- (10^7 PFUs) infected mice. Graphs show mean ± SEM; each point represents an individual mouse. (*F*) Absolute numbers of Nβ neutrophils in MLNs and spleen at 6 h postinfection of NYVAC-C– or NYVAC-C Δ3-injected mice. Boxes with dashed lines indicate absolute number in PBS-injected mice. Graphs show mean ± SEM; each point represents an individual mouse. (*G*) Percentages of Nα (black) and Nβ (green) neutrophils positive for CD11c, MHC II, and CD86 markers. Graphs show mean ± SEM; each square represents an individual mouse. (*H*) Layouts and median fluorescence intensity (MFI) of indicated markers in Nα (black) and Nβ (green) neutrophils at 6 h postinfection in NYVAC-C Δ3-injected mice. In gray is the isotype control. Columns show mean ± SEM of five mice. (*I*) Total numbers of CD8 T cells per spleen secreting IFN-γ and/or TNF-α and/or IL-2 after stimulation with thioglycolate-induced neutrophils, with virus-induced Nα, with virus-induced Nβ-enriched, or with RPMI. Columns show mean ± SEM of triplicates. (*J*) Nβ:Nα ratio in peritoneal cavity at 6 h postinfection in MVA-WT–, MVA-C–, NYVAC-C–, or NYVAC-C Δ3- (10^7 PFUs) injected mice. Graphs show mean ± SEM; each point represents an individual mouse. Data are representative of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

To demonstrate the APC potential of Nα and Nβ neutrophils, both populations were sorted for Ly6G$^+$ (purity, >96%) and cell size and then incubated 16 h with isolated spleen CD8 T cells (purity, >97%; ratio, 1:20) from mice that received i.p. injections of NYVAC or PBS 90 d earlier. Ly6G$^+$ neutrophils (purity, >96%) sorted from i.p. thioglycolate-injected mice were used as controls. We measured antigen-specific CD8 T-cell activation by intracellular cytokine staining (ICS) of IL-2, IFN-γ, and TNF-α. Nα cells alone did not induce antigen-specific CD8 T activation (Fig. 4*I*): The number of cytokine-secreting CD8 T cells was similar to thioglycolate-induced neutrophils (<0.01% of total CD8 T cells). The Nβ-enriched fraction induced a significant increase in CD8 T-cell activation compared with Nα alone (Fig. 4*I*). The CD8 T-cell response in PBS-injected mice was subtracted for each group; this response was <0.01% of total CD8 T cells, indicating that the neutrophils did not activate naïve CD8 T cells. These data indicate that Nα cells alone are unable to activate CD8 T cells and that the presence of Nβ cells is sufficient to activate antigen-specific CD8 T cells, suggesting that Nβs have an APC role.

Given the distinct roles of Nα and Nβ in CD8 T-cell activation, we analyzed the Nβ/Nα ratio in the peritoneal cavity of poxvirus-infected mice. In NYVAC-C Δ3-infected mice, this ratio was significantly higher than in NYVAC-C–infected mice, and also compared with MVA-WT– and MVA-C–infected mice (Fig. 4*J*). The total neutrophil percentage in MVA-WT–, MVA-C–, or NYVAC-C Δ3-infected mice was similar and significantly higher than in NYVAC-C–infected mice (Fig. S4*B*).

NYVAC-C Δ3 Enhances the HIV-Specific T-Cell Response Through Neutrophil Migration. To study the ability of NYVAC-C Δ3 to induce a specific T-cell response to HIV-1 antigens, we used a DNA intramuscular prime/poxvirus i.p. boost approach. This heterologous immunization protocol is more immunogenic than a homologous combination in activating T-cell responses to HIV-1 antigens (27).

BALB/c mice were immunized (*Materials and Methods*), and adaptive T-cell immune responses were measured by ICS of spleen cells. To study the HIV-1–specific T-cell response, splenocytes from infected mice were stimulated with HIV-1 Env-1, Pol-1, or Pol-2 peptides, which are the most immunogenic MHC class I-restricted cytotoxic T lymphocytes (CTLs) peptides of HIV clade C in BALB/c mice (29) or with a pool of overlapping Gag peptides. Alternatively, to study CD8 and CD4 T-cell responses to Env and GPN antigens, splenocytes were pulsed with A20 cells previously nucleofected with the mammalian expression plasmids pcDNA-gp120 (Env) or pcDNA-GPN. To determine the functional profile of CD4 and CD8 T cells, we measured levels of IFN-γ, TNF-α, and IL-2 as well as of lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) as a surrogate marker for induction of killing. We determined the magnitude of the T-cell response as the number of T cells that expressed IFN-γ and/or TNF-α and/or IL-2 and/or CD107a and the polyfunctionality of the response as T cell's capacity to express more than one of these activation markers (13).

NYVAC-C Δ3 induced a significantly higher specific CD8 T-cell response to HIV intracellular antigens than NYVAC-C, both when Pol and Gag antigens were analyzed separately (Fig. 5*A*) and when the overall GPN response was studied (Fig. 5*B*). There were no significant differences between both viruses in the magnitude of the specific CD8 T-cell response to the Env extracellular antigen, using either Env-1 peptide (Fig. S5*A*) or A20-ENV$^+$ (Fig. S5*B*). Neither virus induced a GPN-specific CD4

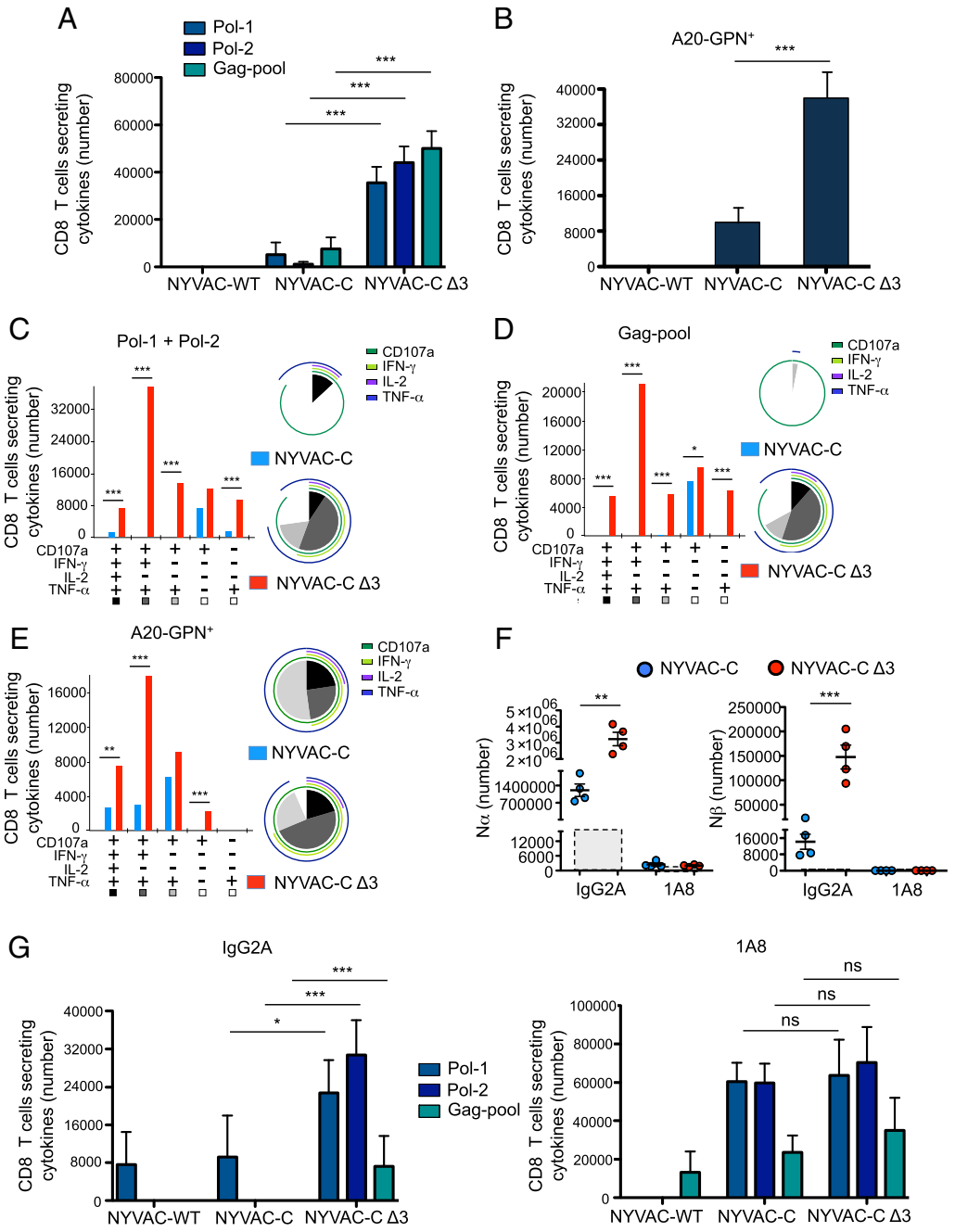

Fig. 5. NYVAC-C Δ3 enhances the magnitude and polyfunctionality of the CD8 T-cell response to HIV-1 Gag–Pol antigens. Vaccine-induced HIV-1–specific CD8 T-cell response in mice ($n = 4$ per group) infected with 10^7 PFUs of NYVAC-WT, NYVAC-C, or NYVAC-C Δ3. The response was measured 11 d after the last immunization, after splenocyte stimulation with HIV-1 peptides/pools or with A20 GPN$^+$. Total value (magnitude) is the sum of total CD8 T cells per spleen that secrete IFN-γ and/or TNF-α and/or IL-2 and/or CD107a. (*A*) Magnitude of Pol-1–, Pol-2–, or Gag pool-specific CD8 T-cell response. Graphs show mean ± CI. (*B*) Magnitude of CD8 T-cell response to A20 GPN$^+$. Graphs show mean ± CI. (*C*) Functional profile of adaptive Pol-1 + Pol-2–specific CD8 T cells. (*D*) Functional profile of adaptive Gag pool-specific CD8 T cells. (*E*) Functional profile of adaptive GPN-specific CD8 T cells. Combinations of responses (*x* axis) and total numbers of functionally distinct cell populations (*y* axis) are shown. Responses are grouped and color-coded based on the number of functions. Pie chart colors indicate the percentage of cytokine-producing cells based on number of functions (inside) and the different activation markers (outside). (*F*) Absolute number of Nα and Nβ neutrophils in the peritoneal cavity at 6 h postinfection in NYVAC-C– or NYVAC-C Δ3- (10^7 PFUs) injected and IgG2A-pretreated or 1A8-pretreated mice. Boxes with dashed lines indicate absolute number in PBS-injected mice. Graphs show mean ± SEM; each point represents an individual mouse. (*G*) Vaccine-induced HIV-1–specific CD8 T-cell response in mice ($n = 4$ per group) infected with 10^7 PFUs NYVAC-WT, NYVAC-C, or NYVAC-C Δ3 and IgG2A-pretreated or 1A8-pretreated. The response was measured 11 d after the last immunization, after splenocyte stimulation with HIV-1 peptides/pools. Total value (magnitude) is the sum of total CD8 T cells per spleen that secrete IFN-γ and/or TNF-α and/or IL-2 and/or CD107a. Graphs show mean ± CI. Data are representative of three independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

T-cell response when stimulated with A20-GPN$^+$. We detected no differences in Env-specific CD4 T-cell response with A20-Env$^+$ between two-virus–infected mouse groups (Fig. S5*C*).

The quality of the Gag and Pol responses, defined as cytokine production and cytotoxic potential, showed that compared with the parental strain, the triple deletion mutant induced a marked

increase in the CTL polyfunctional profile (Fig. 5 *C* and *D*). The CD8 T-cell subset that produced IFN-γ, TNF-α, and CD107a was the most representative population induced with Pol and Gag peptides (Fig. 5 *C* and *D*) and with A20-GPN$^+$ (Fig. 5*E*) in triple deletion mutant-infected mice. This CD8 T-cell subset marks the difference in the CTL polyfunctional profile between two-virus–infected mice (Fig. 5 *C*–*E*, pie charts). These results indicate that the triple deletion mutant enhances the magnitude and the polyfunctional profile of specific CD8 T cells to HIV-1 GPN intracellular antigens.

We also used a DNA intramuscular prime/poxvirus intramuscular boost to immunize and measured adaptive CD8 T-cell immune responses by ICS in spleen cells. NYVAC-C Δ3 induced a significantly higher specific CD8 T-cell response to HIV-1 Pol antigen than NYVAC-C; no significant difference was found between two groups in the response to Gag antigen (Fig. S5*D*).

To test whether neutrophils are involved in the enhanced HIV-specific CTL response, we depleted these cells in vivo using anti-Ly6G mAb 1A8 administered 24 h before virus boost (30). Nα and Nβ populations were depleted similarly in 1A8-pretreated/virus-infected mice compared with isotype control IgG2A-pretreated/virus-infected mice (Fig. 5*F*). The difference in magnitude of the Pol-1–, Pol-2–, and Gag pool-specific CD8 T-cell responses between viruses was greatly reduced in 1A8-pretreated mice (Fig. 5*G*) compared with the significant differences in IgG2A-pretreated mice between two groups (Fig. 5*G*).

The increase in HIV antigen-responsive CD8 T cells in mAb 1A8-pretreated/NYVAC-C Δ3-infected mice compared with mAb IgG2a-pretreated/NYVAC-C Δ3-infected mice might be due to the greater monocyte activation (based on CD11b and CD62L overexpression) in 1A8- than in IgG2a-pretreated mice (Fig. S5*E*). Monocyte numbers in 1A8-pretreated/NYVAC-C Δ3-infected mice were significantly lower compared with IgG2A-pretreated/NYVAC-C Δ3-infected mice, suggesting neutrophil-dependent monocyte migration (Fig. S5*F*). We found no differences in DC activation markers (CD80, CD86) or in DC number between the two groups (Fig. S5 *G* and *H*). These data suggest that neutrophils are involved in the increase in HIV-1 Gag- and Pol-specific CD8 T-cell responses after infection with the triple deletion mutant and that other immune cells induce higher antigen-specific CD8 T-cell responses in the nonphysiological absence of neutrophils.

Discussion

Here we define the biological contribution of the deletion of three VACV inhibitors of the NFκB signaling pathway in the NYVAC genome. The A52, K7, and B15 early proteins, members of the B-cell lymphoma-2 family (31, 32), are involved in suppression of host immune responses (2). During infection, A52 and K7 interact with TNF receptor-associated factor 6 and inhibit its kinase activation capacity (24, 33), whereas B15 binds the IκB kinase complex to inhibit IκBα phosphorylation and degradation (20). In this study, we focused on the A52, K7, and B15 proteins; using single, double, and triple deletion mutants, we demonstrate that the presence of only one of the three inhibitory molecules is sufficient to abolish NFκB activation, ruling out synergy between these proteins as a mode of action. We establish that combined deletion of *A52R*, *K7R*, and *B15R* is necessary for efficient triggering of the NFκB pathway and neutrophil recruitment.

Neutrophils treated with GM-CSF and/or other cytokines can up-regulate MHC class II and the costimulatory molecules CD80/CD86 (APC markers) and promote T-cell activation (11, 12). Neutrophils can acquire macrophage (34) or dendritic phenotypes (10), and such hybrid neutrophil populations with APC-like properties participate in adaptive immune responses (10). In the context of a tumor, the neutrophil subsets generated in the cytokine/chemokine environment can affect tumor growth by influencing CD8 T-cell activation (35). In our study, during NYVAC-C Δ3 infection, we observed recruitment of Nα and Nβ neutrophil subsets as a consequence of the cytokine/chemokine profile produced. Nβ neutrophils are more lobulated, larger, and morphologically more complex; display an enhanced activation profile; have higher levels of APC markers (CD11c, CD80, and CD86); and have greater capacity to induce antigen-specific T-cell activation than Nα cells.

MVA induces NFκB activation (23) and robust neutrophil recruitment after intranasal (36) or intradermal infection (37). We demonstrate that, after i.p. infection, the percentage of neutrophils in MVA-WT–, MVA-C–, or NYVAC-C Δ3-infected mice was similar and that the Nβ/Nα ratio is significantly higher in NYVAC-C Δ3- than in MVA-infected mice. The NYVAC Δ3 vector might thus offer an advantage in generating antigen-specific CD8 T-cell responses compared with the MVA vector.

Attenuated VACV vectors are considered vaccine candidates; specifically, NYVAC, MVA, and ALVAC poxvirus strains are used against emerging infectious diseases and cancer in humans (1). For HIV/AIDS VACV vectors, several strategies have been developed to improve immunogenicity to HIV antigens, such as use of costimulatory molecules, administration of heterologous vectors, and deletion of immunomodulatory viral genes (38). These approaches have yielded promising results in primates and elicited protection after challenge with simian immunodeficiency virus (39), although effectiveness was limited in the RV144 phase III HIV/AIDS human clinical trial (40). In this context, NYVAC-C Δ3 could be considered a valid prototype for future vaccines due to its ability to activate NFκB, to induce specific neutrophil migration, and to enhance CD8 T-cell immune responses to Gag and Pol antigens.

Gag and Pol are the best conserved HIV-1 proteins (41) and are able to shift the CTL response from variable Env epitopes to the conserved Gag and Pol epitopes in the first years of HIV-1 infection (42). In untreated chronic HIV-1–infected individuals, a Gag CTL response correlates with lower HIV viral loads, decreasing HIV viremia (43); furthermore, it correlates with decreasing viremia in early HIV-1–infected patients with suspension of retroviral therapy (42). A prophylactic vaccine that induces a Gag CTL response was recently shown to control simian immunodeficiency virus infection (44). Because the Gag/Pol-specific CD8 T-cell response in NYVAC-C Δ3-infected mice is mainly polyfunctional compared with that of NYVAC-C and because most human HIV nonprogressors preferentially maintain highly functional HIV-specific CD8 T cells (45), the NYVAC-C Δ3 vaccinia vector might constitute a promising approach for prophylactic and therapeutic treatment. By triggering enhanced NFκB activation and specific neutrophil recruitment, the triple deletion mutant could offer a considerable advantage over current NYVAC-based vectors being tested in phase I prophylactic and therapeutic clinical trials (46).

In contrast to Gag and Pol responses, NYVAC-C Δ3 does not induce a significant increase in the CD8 T-cell response to Env compared with NYVAC-C. This difference in antigen response probably depends on Env extracellular secretion compared with Gag–Pol intracellular production. Our data suggest greater neutrophil involvement in the engulfment of VACV-infected cells that express intracellular Gag and Pol than of a secreted antigen such as Env, which could be sequestered by macrophages normally found in the peritoneal cavity.

Neutrophils reportedly have difficulties in priming CD4 T cells compared with CD8 T cells after vaccinia infection (13); in our model, we observe no CD4 T-cell response to GPN in NYVAC-C– or in NYVAC-C Δ3-infected mice.

After migrating to the lymph nodes, neutrophils can compete with classic APCs (DCs, macrophages) to present antigens (47), and a direct interaction between antigen-pulsed neutrophils and CD8 T cells has also been proposed (9). In contrast, VACV-infected neutrophils cannot induce a CD8 T-cell proliferative response in BM in the absence of myeloid APCs (13). When mice were NYVAC-GFP– or NYVAC-GFP Δ3-infected, we did not detect GFP$^+$-infected neutrophils in secondary lymphoid

organs, which would suggest indirect neutrophil infection. Based on this evidence, we speculate that antigen transport results from neutrophil uptake of apoptotic cells in which the antigen has already been processed. The neutrophils that transport processed antigens are able to prime CD8 T cells directly, which then respond/proliferate specifically.

In summary, here we identify a mechanism by which the VACV vector NYVAC lacking *A52R*, *K7R*, and *B15R* genes triggers selective immune responses (Fig. 6). Infection with NYVAC-C Δ3 led to activation of the NFκB signaling pathway in vitro and in vivo, with increased cytokine/chemokine expression followed by specific neutrophil activation and recruitment. This enhanced neutrophil recruitment during infection correlated with an increased CD8 T-cell adaptive immune response specific for heterologous antigens. Further analysis of neutrophil populations identified an activated Nβ cell subtype with APC function that migrated to MLNs and to the spleen. Based on these inherent properties, the NYVAC triple deletion mutant demonstrates a new mechanism for poxvirus-induced immune response that provides a novel basis for virus vaccine vector design.

Materials and Methods

Mice and Injections. BALB/c and C57/BL6J mice (6–8 wk old) were purchased from Harlan. C. Combadière (INSERM, Paris) provided C57/BL6J CCR1 KO mice, and S. Mañes (Centro Nacional de Biotecnología, Madrid) provided C57/BL6J CCR5 KO mice. NYVAC-WT and NYVAC-C vectors were provided by Sanofi Pasteur, and their biological properties have been described (27). For the heterologous DNA prime/NYVAC boost immunization protocol to assay NYVAC virus immunogenicity, mice received 100 μg DNA-C (50 μg plasmid construct pcDNA-$_{CN54}$GP120 + 50 μg plasmid construct pcDNA-$_{CN54}$GPN) or 100 μg DNA-ϕ (100 μg plasmid construct pcDNA) by the intramuscular route (27). Plasmids were purified using the Endofree Plasmid Mega Kit (Qiagen). After 2 wk, mice were immunized i.p. or i.m. with 10^7 PFUs of NYVAC-WT, NYVAC-C, or NYVAC-C Δ3. Mice immunized with sham DNA (DNA-ϕ) followed by a NYVAC-WT boost were used as controls. For in vivo neutrophil depletion, mice were injected i.v. with 200 μg 1A8 mAb (BioXcell) or 200 μg Isotype Control IgG2A mAb (Abcam) 24 h before the boost immunization. Animal studies were approved by the Ethical Committee of Animal Experimentation of the Centro Nacional de Biotecnologia/Consejo Superior de Investigaciones Cientificas in accordance with national and international guidelines and the Royal Decree (RD 1201/2005) (permits 12013 and 13013).

Cells. African green monkey kidney cells (BSC-40; American Type Culture Collection) and primary chicken embryo fibroblast (CEF) cells (Intervet) were grown in DMEM supplemented with 10% (vol/vol) FCS. Human monocytic THP-1 cells (ATCC), murine macrophage J774.G8 cells (ATCC), and A20 cells (ATCC) were cultured in RPMI medium 1640 (Sigma-Aldrich) supplemented with 50 μM 2-mercaptoethanol and 10% (vol/vol) FCS. THP-1 cells were differentiated into macrophages by treatment with 5 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 24 h before use. Murine macrophage cells were obtained from peritoneal exudates 4 d after i.p. administration of 1 mL 9% (wt/vol) casein; total cells were cultured (12 h) in RPMI 1640 with 10% (vol/vol) FCS, and nonadherent cells were removed. Cells were cultured in a humidified air 5% (vol/vol) CO_2 atmosphere at 37 °C.

Viruses. The poxvirus strains used included the genetically attenuated VACV-based vector NYVAC-WT and the recombinant NYVAC-C (27). Deletion

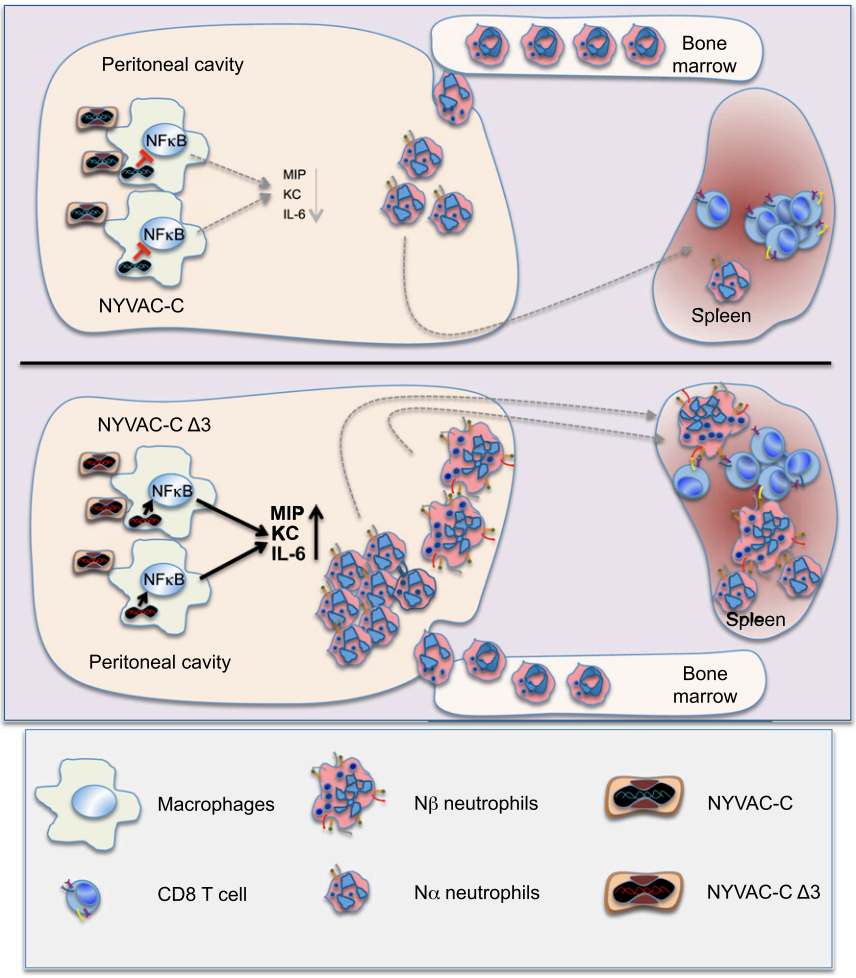

Fig. 6. NYVAC-C Δ3 capacity to increase neutrophil peritoneal recruitment and spleen migration compared with NYVAC-C.

mutants were constructed using Discosoma sp. (ds)Red2 and red shifted (rs)GFP as fluorescent markers. BSC-40 cells were infected with 0.01 PFU per cell of NYVAC-C (1 h) and transfected with pGem-RG-*A52R*-wm, pGem-RG-*B15R*-wm, or pGem-RG-*K7R*-wm plasmids using Lipofectamine (Invitrogen) to generate single, double, and triple deletion mutants. Deletion mutants were selected from progeny virus by consecutive rounds of plaque purification as described (48). NYVAC-GFP and NYVAC-GFP Δ3 were constructed using rsGFP as a fluorescent marker and β-galactosidase as a reporter gene. BSC-40 cells were infected with 0.01 PFU per cell of NYVAC-C or NYVAC-C Δ3 (1 h) and transfected with VACV insertional plasmid vector pLZAW1-LEO (49) to replace the HIV cassette with the GFP gene. Recombinant viruses were selected from progeny virus by consecutive rounds of plaque purification as reported (27). NYVAC-C Δ3 K7-rev was constructed using the K7R gene and β-glucuronidase as the reporter gene. BSC-40 cells were infected with 0.01 PFU per cell of NYVAC-C Δ3 (1 h) and transfected with VACV insertional plasmid vector pCAR-2/K7R to insert the *K7R* gene into the HA locus. Recombinant viruses were selected from progeny virus by consecutive rounds of plaque purification as reported (50). In vitro virus infections were performed with 2% (vol/vol) FCS. All viruses were grown in primary CEF cells, similarly purified through two 36% (wt/vol) sucrose cushions, and virus titers were determined by immunostaining plaque assay in BSC-40 cells as described (51).

Plasmids. The plasmid transfer vectors pGem-RG-*A52R*-wm, pGem-RG-*B15R*-wm, and pGem-RG-*K7R*-wm, used for deletion of *A52R*, *B15R*, and *K7R* ORF from the NYVAC-C genome, respectively, were obtained by sequential cloning of *A52R*, *B15R*, and *K7R* recombination flanking sequences into the plasmid pGem-Red-GFP-wm as described (52). The plasmid transfer vector pCAR-2/K7R used for the insertion of the K7R gene was obtained by cloning the *K7R* sequence into the plasmid pCAR-2 (patent WO2007132052 A1).

The NYVAC genome was used as the template to amplify the left flank, the repeated left flank and the right flank of the three genes, or the sequence of *K7R*; the oligonucleotides used are listed in Table S1. The resulting plasmids pGem-RG-*A52R*-wm, pGem-RG-*B15R*-wm, pGem-RG-*K7R*-wm, and pCAR-2/K7R were confirmed by DNA sequence analysis.

PCR. Viral DNA was extracted by the SDS-proteinase K-phenol method. Primers LF´A52R-Eco and RFA52R-Bam (spanning *A52R* flanking regions), LF´B15R-Aat and RFB15R-Bam (spanning *B15R* flanking regions), and LFK7R-Aat and fi-FIL-BR (TTATAGGATCCCTCCAGGAGAAAG) (spanning *K7R* flanking regions) were used for PCR analysis of *A52R*, *B15R*, and *K7R* loci, respectively. Primers TK-L and TK-R were used for PCR analysis of the TK (thymidine kinase) locus as described (53) to confirm the replacement of the HIV cassette with the GFP gene. Primers HA1 (GTCACGTGTTACCACGCA) and HA2-NYVAC–reverse (CCGAGTAAGGCATTAGG) were used for PCR analysis of the HA locus to confirm insertion of the *K7R* gene.

RT-PCR. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and was treated with DNase I recombinant, RNase-free. Subsequently, first-strand cDNA was synthesized with Oligo $(dT)_{12–18}$ primers (Invitrogen) using SuperScriptIII Reverse transcriptase (Invitrogen) following the manufacturer's protocol, and cDNA was used for PCR amplification. The primers K7RinternalFwd-Bam and K7RinternalRev-Not used for amplification of *K7R* cDNA were the same as for cloning and are described in Table S1.

Western Blot. BSC-40 cells were lysed in Laemmli buffer, and extracts were fractionated by 8% (vol/vol) SDS/PAGE and analyzed by Western blot using rabbit polyclonal anti-gp120 (Centro Nacional de Biotecnología), -gag p24 (ARP 432, NIBSC, Centralised Facility for AIDS Reagents), or -E3 antibodies (Centro Nacional de Biotecnología). J774.G8 and THP-1 cells were lysed in Cell Lysis Buffer (Cell Signaling) supplemented with PMSF, and extracts were fractionated by 10% (vol/vol) SDS/PAGE and analyzed by Western blot using anti-phospho IκBα, -IκBα, and -tubulin (Cell Signaling). Nuclear extracts were obtained using a Nuclear/Cytosol Fractionation Kit (BioVision Research) and analyzed by Western blot using anti-H2A mAb (Cell Signaling).

EMSAs. For gel shift assays, nuclear extracts were incubated with 0.5 ng ^{32}P-end–labeled double-stranded oligonucleotide probe 5′-AGTTGAGGGGACTTTCCCAGGC-3′, which has NFκB binding sites. The binding reaction was performed in binding buffer with 1.5 mg poly (dI-dC), and binding products were resolved by electrophoresis.

Immunofluorescence. Cells were washed with PBS, fixed with 4% (vol/vol) paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 20% (vol/vol) FCS. Cells were coincubated with polyclonal anti-p65 (Santa Cruz) and -E3 antibodies (Centro Nacional de Biotecnología). Bound primary antibodies were detected with AlexaFluor488 or 594-conjugated antibodies (Invitrogen). Cell nuclei were DAPI-stained (Sigma). The model of the microscope was LEICA TCS SP5 multispectral confocal system; type, magnification, and numerical aperture of the objective lenses were HCX PL APO lambda blue 63.0×/1.4 oil immersion; the temperature was 25 °C; the imaging medium was Prolong Gold (Invitrogen); and the acquisition software was Leica LASAF version 2.6.0.

Cytokine/Chemokine Analysis. Sera and peritoneal washes from PBS- or virus-injected mice, as well as supernatants of mock- or virus-infected J774 and peritoneal macrophages, were assayed to detect cytokines and chemokines in multiplex analyses using LuminexXMAP technology. IL-8 levels from sera, peritoneal washes, and supernatants of J774 macrophages were quantified by IL-8 ELISA Kit (MyBioSource).

In Vitro Migration Assay. Migration assays were performed in 6.5-mm-diameter transwell dishes (Corning Costar) with 3-μm pore filters. Murine BM cells were added to the upper chamber, and supernatants of mock- or virus-infected J774 cells were added to the bottom chamber. Migration in medium alone served as the negative control. After 3 h of incubation, cells in the medium of the bottom chamber were collected and passed for 1 min at a high flow rate in a LSRII cytometer (BD). The number of cell events was counted, and remaining cells in the tube were stained with anti-Ly6G (1A8) and -CD11b (M1/70) (BD).

Apoptosis Assay. The annexin V binding assay was performed in combination with propidium iodide (PI) to monitor the integrity of the neutrophil membrane.

Hematoxylin/Eosin Staining. $Ly6G^+$ cells were fixed with acetone for 10 min and then stained.

Peptides. The HIV-1 peptides Env-1 (sequence, PADPNPQEM), Pol-1 (LVGPTPVNI), and Pol-2 (YYDPSKDLI) were described as H-2^d–restricted CTL epitopes (29) and were provided by the CNB-CSIC Proteomics Service. The HIV-1 Gag pool (60 peptides of Gag-1 + 61 peptides of Gag-2) and Nef pool (49 peptides) were provided by the EuroVacc Foundation and have been described (27); they span the entire HIV-1 Gag and Nef regions included in the immunogens as consecutive 15 mers with 11-amino acid overlaps.

Nucleofection Assay. A20 cells were nucleofected using 4D-Nucleofector (Lonza) and the Amaxa SF Cell Line Kit (Lonza). Cells were nucleofected with 6 μg pcDNA-$_{CN54}$gp120 or pcDNA-$_{CN54}$GPN; pcDNA-ϕ was used as the negative control. At 24 h postnucleofection, A20 cells were used to restimulate splenocytes from infected mice (1:10, A20–splenocyte ratio).

Cell Isolation. $CD8^+$ T cells were purified from spleen using the Dynabeads FlowComp Mouse CD8 Kit (Invitrogen). $Ly6G^+$ cells were sorted using MoFlo XDP sorter (Beckman Coulter).

Flow Cytometry. For ICS, erythrocyte-depleted splenocytes were rested overnight and resuspended in RPMI 1640 with 10% (vol/vol) FCS and 1 μg/mL Golgiplug (BD), monensin (eBioscience), and anti-CD107a (1D4B) (BD). After restimulation with peptides or A20 cells [6 h, 37 °C, 5% (vol/vol) CO_2], splenocytes were stained for surface markers with anti-CD3 (145-2C11), -CD4 (GK1.5), and -CD8 (53-6.7) (all from BD); fixed; permeabilized (Cytofix/Cytoperm Kit; BD); and stained intracellularly with anti–IL-2 (JES6-5H4), –IFN-γ (XMG 1.2), and –TNF-α (MP6-X722) (all from BD). PECs, obtained after injection of cold PBS into previously infected mice, were counted and stained with anti-Ly6G (1A8), -CD3 (145-2C11), -CD11b (M1/70), -CD19 (1D3), -CD80 (16-10A1), -CD86 (GL-1), -MHC class I (H-2Kd; SF1-1.1), -MHC class II (1-A/1-E; 2G9) (all from BD), -CD45 (30-F11; Biolegend), -CD11c (N418; eBioscience), -F4/80 (BM8, eBioscience), and -CD62L (MEL-14, Beckman Coulter). Peripheral blood, BM, omentum, spleen, and lymph nodes cells were stained with anti-LyG (1A8), -CD45 (30-F11), -CD11b (M1/70), and -CD115 (AFS98, eBioscience). The dead cells were stained using the violet LIVE/DEAD stain kit (Invitrogen) in all cytometry analyses.

Cells were acquired using a GALLIOS (Beckman Coulter) or LSRII (BD) flow cytometer, and data analyses were performed with FlowJo software version 8.5.3 (Tree Star). Boolean combinations of single functional gates were created with FlowJo to determine the frequency of each response based on all possible combinations of cytokines or of differentiation marker expression.

Statistical Analysis. For statistical analysis of CD8 T-cell response to HIV-1 Gag–Pol antigens, we used an approach that corrects measurements for medium response and allows calculation of confidence intervals (CIs) and *P* values of

hypothesis tests (50). Only antigen response values significantly larger than the corresponding RPMI are shown. Background levels (splenocytes in RPMI) were subtracted from all values used to allow analysis of proportional representation of responses. Analysis and presentation of distribution were performed using SPICE version 5.1, downloaded from exon.niaid.nih.gov. For statistical analysis of T-cell responses to HIV-1 Env antigen, the Mann–Whitney *t* test was used. For statistical analysis of cytokine/chemokine expression and in vitro neutrophil migration, one-way ANOVA was applied to compare all viruses used. Student's *t* test was used for other analyses to establish the differences between two groups.

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Supporting Information

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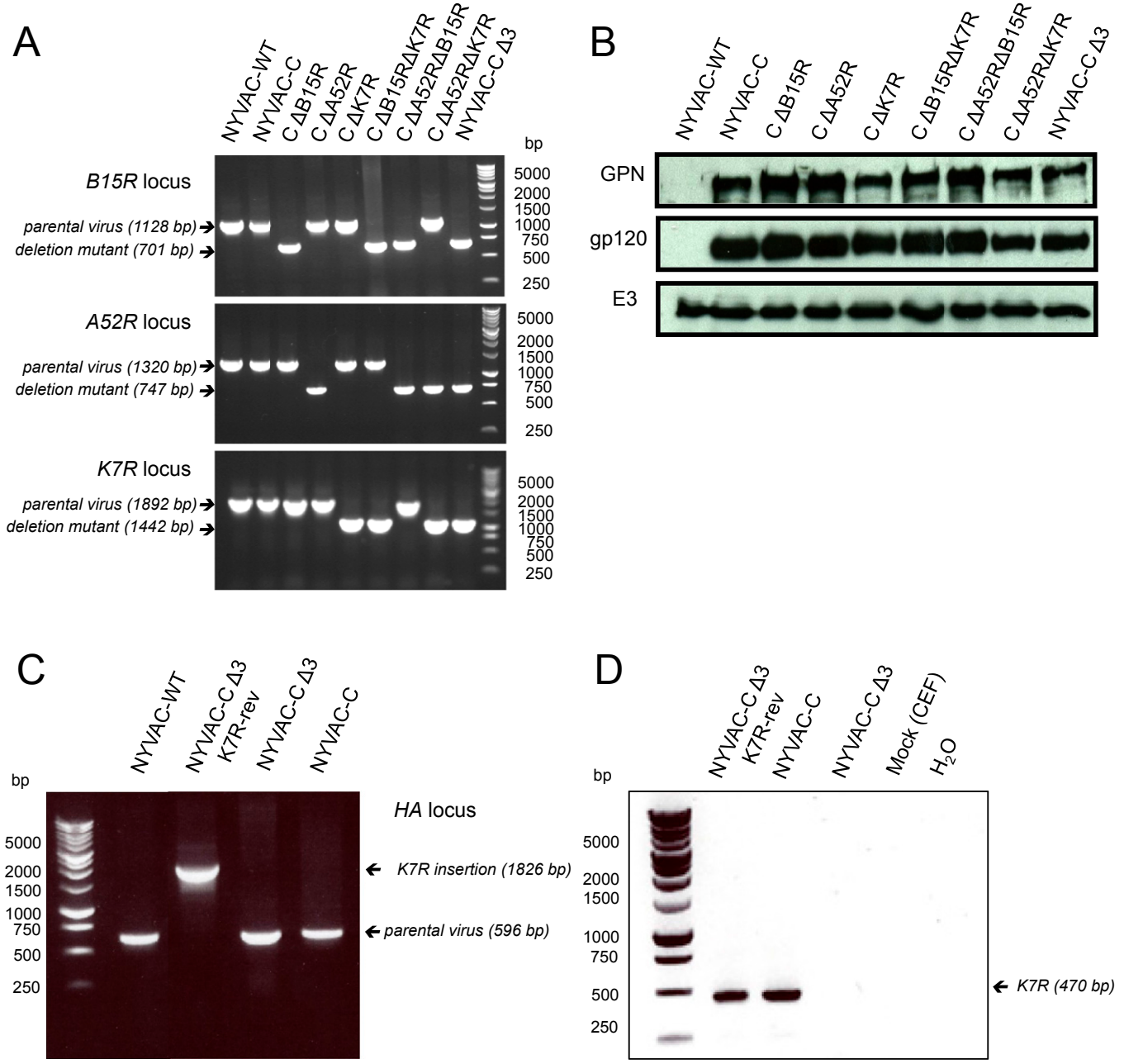

Fig. S1. Generation and characterization of NYVAC-C mutants. (*A*) PCR analysis to confirm *A52R*, *B15R*, and *K7R* gene deletion. Viral DNA was extracted from BSC-40 cells infected with the viruses as indicated (2 PFUs per cell, 24 h). (*B*) Western blot showing expression of HIV-1 antigens and E3 VACV protein in BSC-40 cells infected as in *A*. (*C*) PCR analysis to confirm *K7R* gene insertion. Viral DNA was extracted from BSC-40 cells infected with the viruses as indicated (1 PFU per cell, 24 h). (*D*) RT-PCR analysis to confirm *K7R* gene expression. RNA was extracted from CEF cells infected with the viruses as indicated (1 PFU per cell, 24 h).

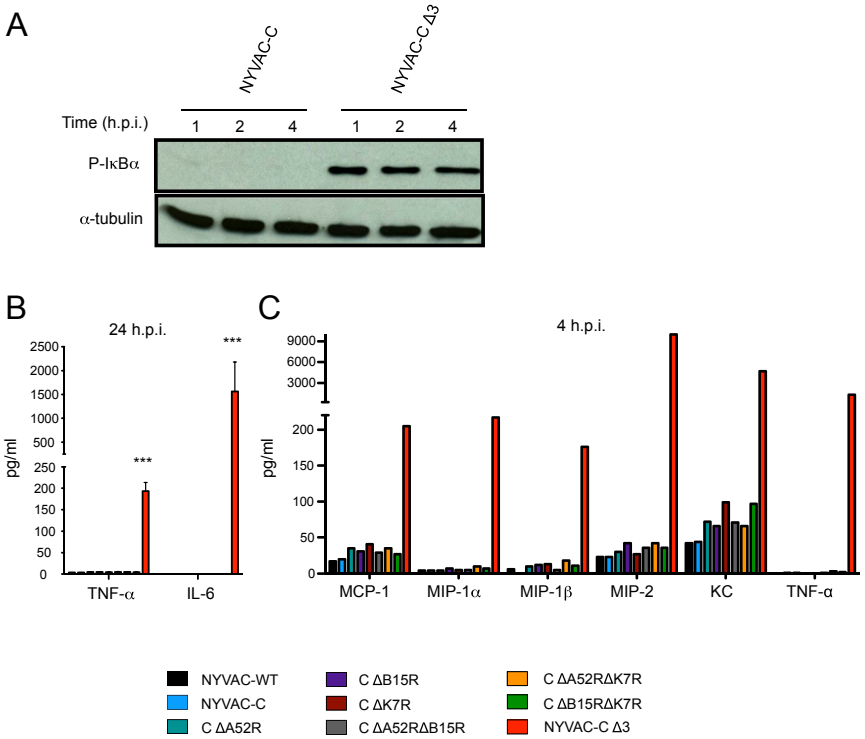

Fig. S2. Activation of NFκB pathway in THP-1 cells and cytokine/chemokine levels in supernatants of JJ74 macrophages and primary peritoneal macrophages after NYVAC infection. (*A*) Phosphorylated IkBα analyzed by Western blot in THP-1 human monocytes, differentiated into macrophages, infected with NYVAC-C or NYVAC-C Δ3 (5 PFUs per cell) for 1, 2, and 4 h. α-tubulin was used as the internal loading control. (*B*) Concentrations of cytokines TNF-α and IL-6 at 24 h post-infection, quantified by immunoassay in supernatants of J774 cells infected as indicated. Values show mean ± SEM of duplicates, representative of three independent experiments. (*C*) Concentration of MCP-1, MIP-1α, MIP-1β, MIP-2, KC, and TNF-α at 4 h postinfection in supernatants of primary peritoneal macrophages infected with indicated viruses. Data are representative of two independent experiments. ***$P < 0.001$.

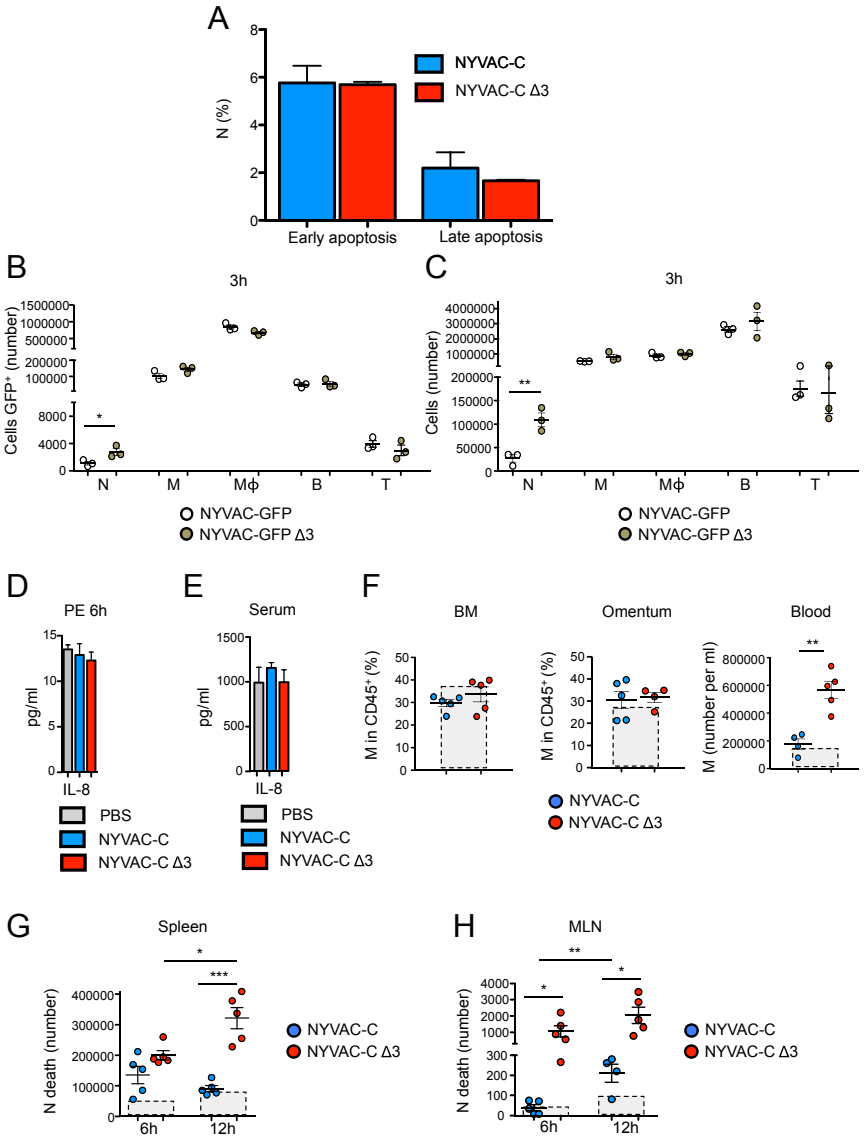

Fig. S3. In vitro and in vivo neutrophil cell death, PEC characterization after NYVAC-GFP or NYVAC-GFP Δ3 infection, and IL-8 secretion and monocyte trafficking after NYVAC-C or NYVAC-C Δ3 infection. (*A*) Percentages of early apoptotic (Annexin $V^+{\cdot}PI^-$) and late apoptotic (Annexin $V^+{\cdot}PI^+$) migrated neutrophils toward supernatants of NYVAC- or NYVAC-C Δ3-infected (4 h) J774 macrophages. Values show mean ± SEM of duplicates. (*B*) Absolute numbers of GFP^+ neutrophils (N), monocytes (M), macrophages (Mϕ), B cells (B), and T cells (T) in the peritoneal cavity of NYVAC-GFP– or NYVAC-GFP Δ3-injected (10^7 PFUs) mice at 3 h postinfection. Each point represents an individual mouse. (*C*) Absolute numbers of neutrophils (N), monocytes (M), macrophages (Mϕ), B cells (B), and T cells (T) in the peritoneal cavity of NYVAC-GFP– or NYVAC-GFP Δ3-injected (10^7 PFUs) mice at 3 h postinfection. Each point represents an individual mouse. Shown are IL-8 levels at 6 h postinfection in peritoneal exudates (*D*) and serum (*E*) of PBS-, NYVAC-C–, or NYVAC-C Δ-3-injected mice ($n = 5$ per group). (*F*) Percentages of monocytes in BM and omentum, and total number of monocytes in blood at 6 h postinfection from NYVAC-C– or NYVAC-C Δ3-injected mice. Boxes with dashed lines indicate absolute numbers of cells in PBS-injected mice. Each point represents an individual mouse. Total numbers of neutrophil cell death in spleen (*G*) and in MLNs (*H*) at 6 and 12 h postinfection from NYVAC-C– or NYVAC-C Δ3-injected mice. Boxes with dashed lines indicate absolute numbers of cell deaths in PBS-injected mice. Each point represents an individual mouse. Graphs show mean ± SEM. Data are representative of two independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

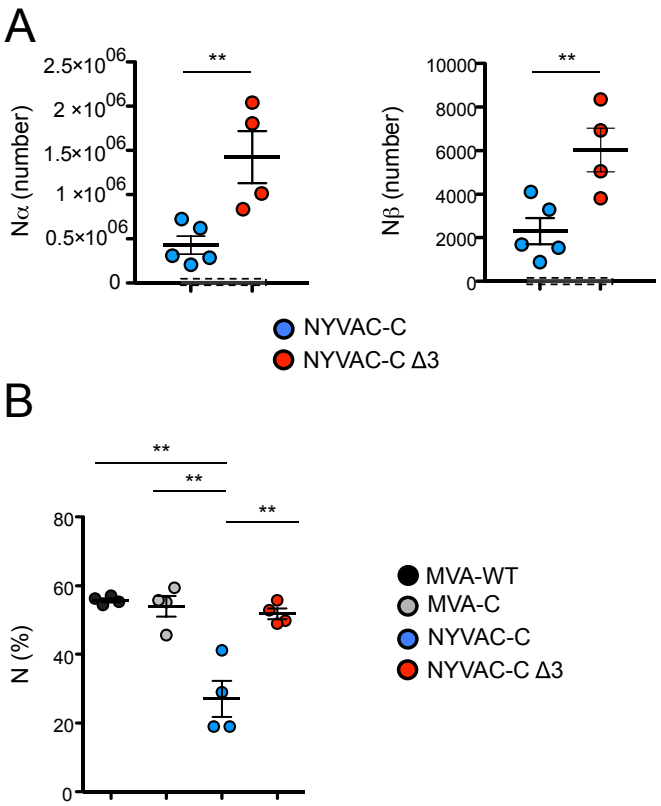

Fig. S4. Nα and Nβ neutrophils in NYVAC-peritoneal exudate-injected mice and percentages of PEC neutrophils in different poxvirus-infected mice. (*A*) Absolute numbers of peritoneal Nα and Nβ neutrophils in NYVAC-C– and NYVAC-C Δ3-peritoneal exudate-injected mice. Boxes with dashed lines indicate percentages in PBS-peritoneal exudate-injected mice. (*B*) Percentages of neutrophils at 6 h postinfection from MVA-WT– or MVA-C–, or NYVAC-C– or NYVAC-C Δ3-injected mice. Graphs show mean ± SEM; each point represents an individual mouse. Data are representative of two independent experiments. $^{**}P < 0.01$.

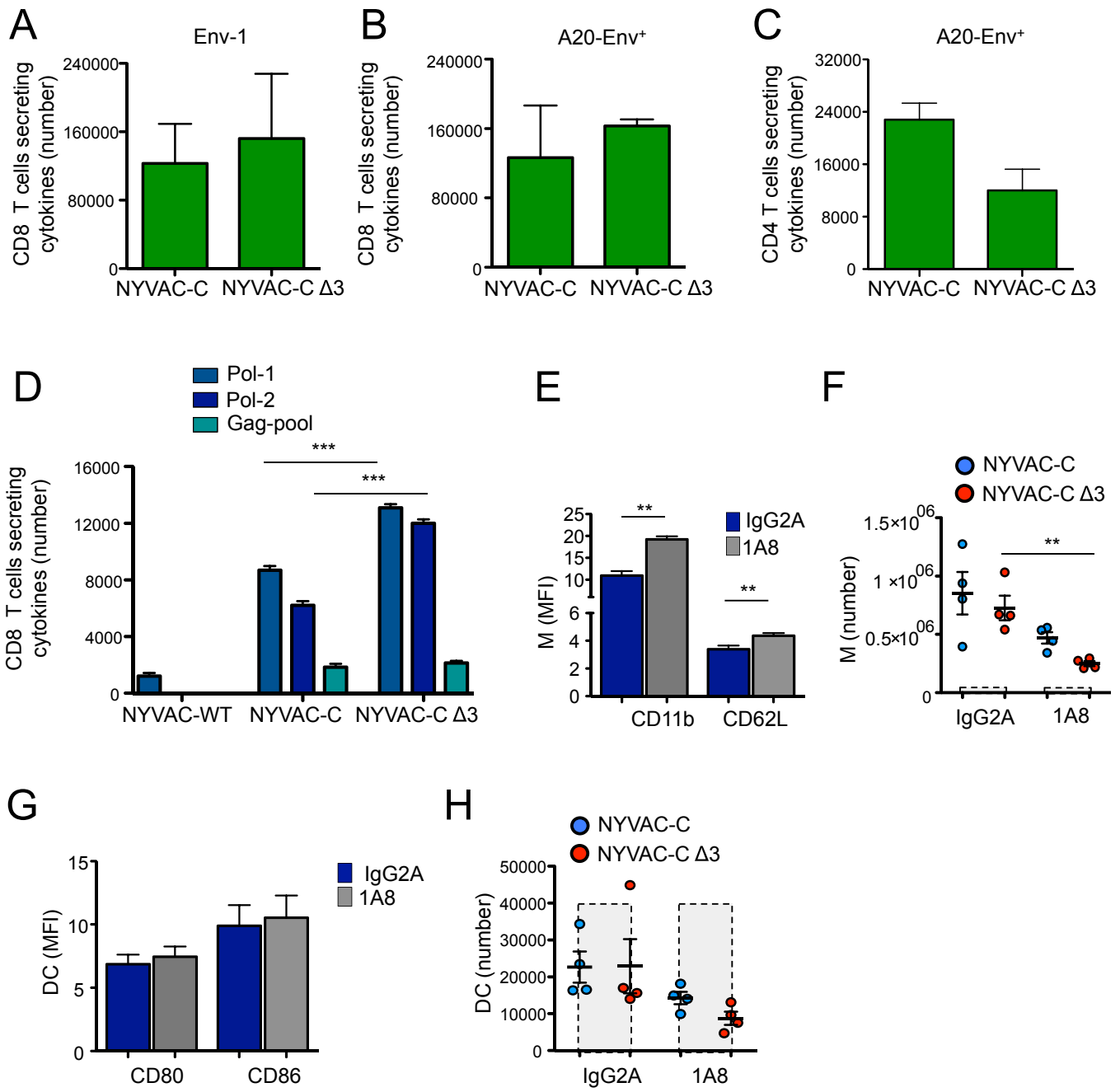

Fig. S5. Magnitude of the specific CD8 and CD4 T-cell response to HIV-1 antigens, and M or DC activation and absolute numbers in 1A8-pretreated/NYVAC-infected mice. Shown is the vaccine-induced HIV-1–specific T-cell response in mice ($n = 4$ per group) infected with 10^7 PFUs of NYVAC-C or NYVAC-C Δ3. The response was measured 11 d after the last i.p. immunization, after stimulation of splenocytes with Env-1 peptide (*A*) or with A20 Env$^+$ (*B* and *C*), or after the last intramuscular immunization and after stimulation of splenocytes with HIV-1 peptides/pools (*D*). Total value (magnitude) is the sum of percentages of CD8 or CD4 T cells per spleen that secrete IFN-γ and/or TNF-α and/or IL-2 and/or CD107a. Values show mean ± SEM of two independent experiments. Shown are the MFIs of indicated markers in monocytes (M) (*E*) and in DCs (*G*) at 6 h postinfection in NYVAC-injected and IgG2A- or 1A8-pretreated mice. Also shown are M (*F*) and DC (*H*) absolute numbers at 6 h postinfection in NYVAC-injected and IgG2A- or 1A8-pretreated mice. Boxes with dashed lines indicate numbers in PBS-injected mice. Graphs show mean ± SEM; each point represents an individual mouse. $^{**}P < 0.01$, $^{***}P < 0.001$.

Table S1. Primers used for the deletion of *A52R*, *B15R*, and *K7R* genes and for the insertion of *K7R* gene

Genes	Flanks of genes	Name of primers	Sequence of primers
A52R	Left	LFA52R-Apa	TATATTGGGCCCTACTACGATTAA
A52R	Left	LFA52R-Sph	CTTTATGCATGCGGTGATCACACA
A52R	Repeated left	LF′A52R-Eco	ATTAGAGAATTCTACGATTAACGA
A52R	Repeated left	LF′A52R-Cla	CTTTATATCGATGGTGATCACACA
A52R	Right	RFA52R-Cla	GGAAATATCGATGAAAGTTAAAAG
A52R	Right	RFA52R-Bam	TCTGCCGGATCCAATGTAGTAATG
B15R	Left	LFB15R-Kpn-F	TTTATAGGTACCTTCTTTGAGGACT
B15R	Left	LFB15R-Kpn-R	GTTGGCGGTACCCTTACCAATTGCA
B15R	Repeated left	LF′B15R-Aat	TTCTTTGACGTCTGTTTTCCTGAAG
B15R	Repeated left	LF′B15R-Xba	GTTGGCTCTAGACTTACCAATTGCA
B15R	Right	RFB15R-Kpn	CGTATGGGTACCTGAGTTGTACATC
B15R	Right	RFB15R-Bam	GTGTCGGGATCCGAATTAGCATATT
K7R	Left	LFK7R-Aat	CAAAGGACGTCATCATCATTTTTTCACC
K7R	Left	LFK7R-Xba	GCTCTAGAGACTATCTCACAAAAG
K7R	Repeated left	LF′K7R-Eco	CGGAATTCATCATCATTTTTTCACC
K7R	Repeated left	LF′K7R-Cla	CCATCGATGACTATCTCACAAAAG
K7R	Right	RFK7R-Cla	CCATCGATTCTAGAAAAAAAAATTGAATTG
K7R	Right	RFK7R-Bam	CGGGATCCAACAAGGGGTTGG
K7F		K7RinternalFwd-Bam	CGCGGATCCATGGCGACTAAATTAG
K7R		K7RinternalRev-Not	AAGGAAAAAAGCGGCCGCTCAATTCAATTTTTTTTC

Restriction enzyme cleavage sites are underlined.

3.2 Summary

3.2.1 New vaccinia virus promoter as a potential candidate for future vaccines

Modified vaccinia Ankara (MVA) vector is being tested as a candidate vaccine to express heterologous pathogen antigens, and is being further improved to potentiate antigen-specific immune responses. In this respect, efforts to develop new MVA vaccine candidates focus on using promoters to improve the timing of antigen expression and thus, increasing immune responses.

Two categories of VACV early genes have been defined, based on their temporal expression. We selected the first temporal early class of genes and performed sequence alignment to define the appropriate promoter consensus sequence of these genes and the spacer between the core sequence and the gene start codon. Using the motif discovery program Multiple EM for Motif Elicitation (MEME), we observed that 82% of the genes studied contain a promoter core that corresponds to a newly defined consensus sequence; 73% of the genes presented this sequence between nucleotides (nt) -70 and -20 upstream of the open reading frame.

With these data, we designed a new synthetic late-early optimized (LEO) VACV promoter that contains the late part of the synthetic VACV pS promoter and the newly defined early consensus sequence, followed by a 38-nt spacer. We compared the strength of the new LEO promoter with the viral synthetic early/late pS promoter, since pS has been used successfully to express high levels of heterologous antigens in poxvirus-based vaccine candidates.

We generated the attenuated recombinant viruses MVA-LEO-GFP and MVA-PS-GFP to define the temporal expression of GFP under the control of the two promoters and to study GFP-specific immunogenicity. In contrast to the synthetic VACV promoter (pS), LEO induced significantly higher *in vitro* GFP expression within the first hour after infection, which correlated with an enhanced GFP-specific CD8 T cell responses *in vivo*, which demonstrates its potential for use in future vaccines.

Short Communication

New vaccinia virus promoter as a potential candidate for future vaccines

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Here we describe the design and strength of a new synthetic late-early optimized (LEO) vaccinia virus (VACV) promoter used as a transcriptional regulator of GFP expression during modified vaccinia Ankara infection. In contrast to the described synthetic VACV promoter (pS), LEO induced significantly higher levels of GFP expression *in vitro* within the first hour after infection, which correlated with an enhancement in the GFP-specific CD8 T-cell response detected *in vivo*, demonstrating its potential use in future vaccines.

Vaccines against intracellular human pathogens such as human immunodeficiency virus (HIV), hepatitis C virus, malaria and leishmania are designed to induce efficient antigen-specific T-cell immune responses that, in some cases, correlate with protection (Gómez *et al.*, 2012b; Good & Doolan, 2010; Sánchez-Sampedro *et al.*, 2012; Vijayan *et al.*, 2012; Yusim *et al.*, 2013). One of the most popular vectors selected to trigger such responses are the poxviruses, and, in particular, highly attenuated vaccinia virus (VACV) strains such as modified vaccinia Ankara (MVA), NYVAC and ALVAC have been used successfully against emergent infectious diseases and cancer in humans (Gómez *et al.*, 2012a).

It is known that the timing of expression of heterologous antigens in the VACV system affects the capacity to induce antigen-specific T-cell immune responses (Baur *et al.*, 2010) since the efficiency with which an antigen is processed and presented on the surface of infected cells influences its recognition (Moutaftsi *et al.*, 2009). Considering that immunodominance is defined as the phenomenon whereby only a small fraction of all the possible epitopes from a particular pathogen elicits an immune response in an infected individual (Pasquetto *et al.*, 2005), it is possible to modulate such immunodominance hierarchy, changing the timing and the quantity of intracellular antigen production (Wilson & Hunter, 2008). In fact, it has been described that, in VACV, 90 % of the most recognized antigens by CD8 T-cells were ranked in the top 50 % in terms of mRNA expression (Sette *et al.*, 2009), and there is a correlation between viral gene expression and immunodominance hierarchy after a second immunization due to a mechanism of cross-competition between T-cells specific for early and late viral epitopes (Kastenmuller *et al.*, 2007).

Recently, after a deep analysis of the VACV transcriptome, two groups have defined two categories of early genes based on their temporal expression (Assarsson *et al.*, 2008; Yang *et al.*, 2010). Assarsson *et al.* (2008), using a genome tiling array approach, differentiated the immediate-early genes from the early genes, whereas Yang *et al.* (2010), using deep RNA sequence analysis, differentiated the E1.1 genes from E1.2 genes as subclusters of early genes and also defined a 15 nt consensus sequence (AAAANTGAAAANNNA) that corresponds to the core of early genes promoter. Both authors underline that there is a difference in the time of expression between the two early classes of genes, but Yang *et al.* (2010) avoided the terminology of immediate-early and early genes because both subclusters (E1.1 and E1.2) were transcribed in the presence of the protein synthesis inhibitor cycloheximide, while the transcription of early genes depends on the synthesis of one or a few immediate-early proteins (Honess & Roizman, 1975; Ross & Guarino, 1997; Salser *et al.*, 1970).

The core promoter of E1.1 genes more closely corresponds to the consensus sequence than those of E1.2 genes, suggesting that this difference could explain the readiness of E1.1 genes to be recognized by the transcription machinery before recognition of E1.2 genes (Yang *et al.*, 2010).

In an effort to select an optimized virus promoter for antigen expression, in this study we selected the first temporal early class of genes (immediate-early and E1.1) described by both groups (Table S1, available in JGV Online), comprising 45 genes in total, and performed a sequence alignment using a region of 200 nt upstream of the translation initiation site in order to define the

One supplementary table is available with the online version of this paper.

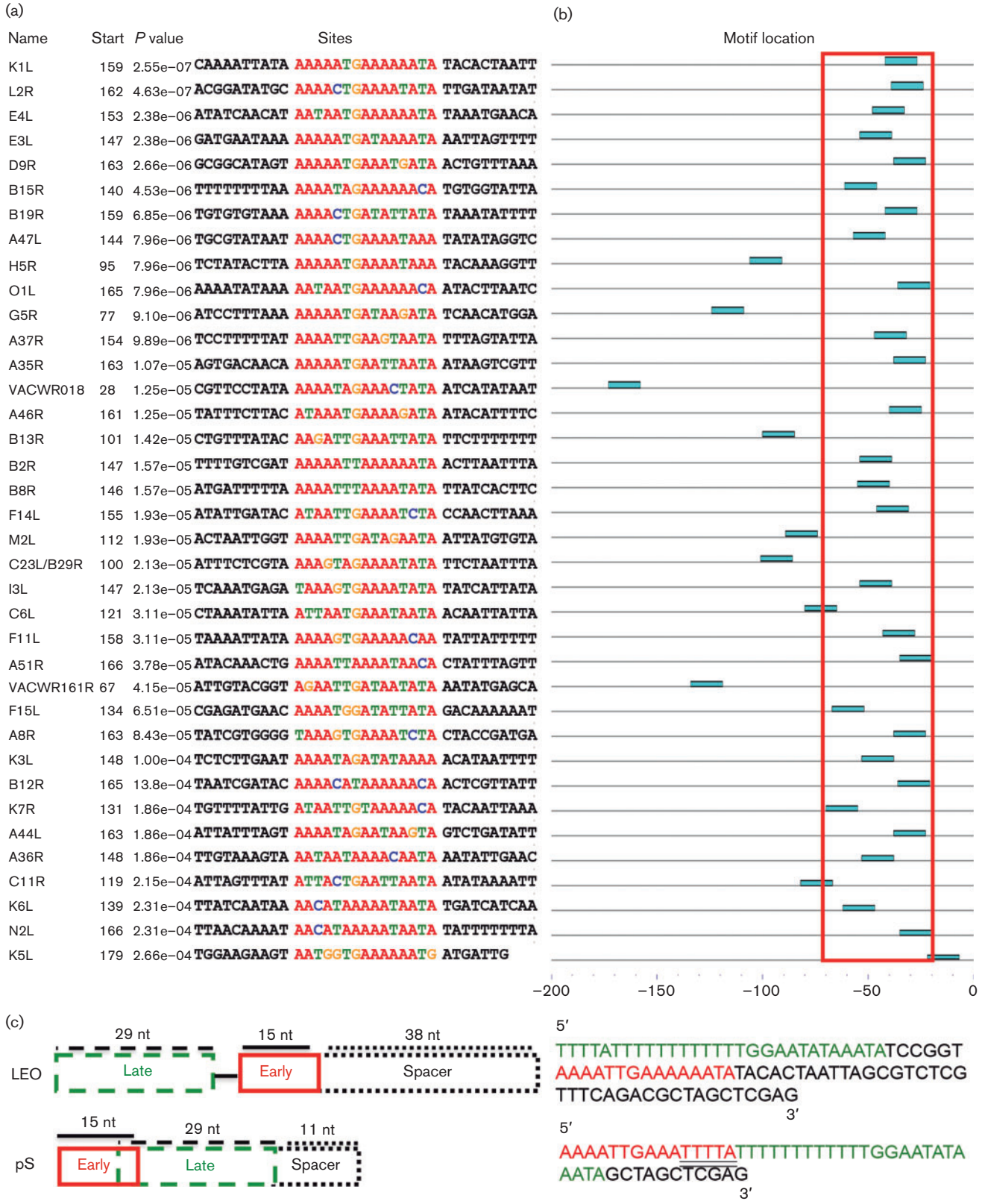

Fig. 1. Design of the synthetic LEO promoter. (a) Alignment of the core promoter sequence of 37 selected early genes using the MEME program. For the analysis, we used the 200 nt upstream of the start codons of the genes. The distribution of motif attributed was zero or unity for the sequence. The maximum width of the motif selected for the analysis was 20 nt. The *P* value

of a site was computed from the match score of the site with the position-specific score matrix for the motif. Only sequences with $P<0.001$ are represented. (b) Alignment of all 37 sequences of 200 nt. The height of the motif 'block' is proportional to −log (*P* value) and the position in the panel corresponds to the motif location. The red box emphasizes the region between −70 and −20 nt upstream of the start codons of the genes. (c) Schematic representation and sequence of LEO and pS promoters. The double solid line represents the nucleotides shared by early and late fractions.

appropriate consensus sequence of the promoter of these genes and to determine the spacer between the core sequence and the start codon of the genes. Using the motif discovery program Multiple Em for Motif Elicitation (MEME), we observed that 82 % of the studied genes (37/45) contained the core of a promoter that closely corresponded to a newly defined consensus sequence, AAAANTGAAAAAATA (Fig. 1a), and in 73 % of them (27/37) the first nucleotide of the core was between positions −70 and −35 and the last nucleotide of the core was between positions −55 and −20 (Fig. 1a, b).

With all these data, we designed a new synthetic late-early optimized (LEO) VACV promoter that contained the same late part present in the widely used synthetic VACV pS promoter (Chakrabarti *et al.*, 1997) prior to the newly defined early consensus sequence, with a T in position 5, and followed by a spacer of 38 nt (Fig. 1c). The purpose of using this LEO promoter in the transcriptional control of a heterologous antigen is to increase the immediate expression of such an antigen during VACV infection. With this aim, we decided to compare the strength of the new LEO promoter with the viral synthetic early/late pS promoter (Chakrabarti *et al.*, 1997), since pS has been used successfully for the expression of high levels of heterologous antigens in vaccine candidates based on poxviruses (Gómez *et al.*, 2007a) and has been described previously as a better promoter than p7.5 and pC11R, and similar to pB8R, pA44L and pF11L in terms of gene expression levels during the first 8 h of infection (Orubu *et al.*, 2012). The pS promoter contains a different early motif that shares 5 nt with the following late part and has a spacer of 11 nt (Fig. 1c).

The new LEO promoter sequence was synthesized by GENEART GmbH (Regensburg) and inserted into the VACV insertional plasmid vector pLZAW1, which contains the right thymidine kinase (TK) flanking arm, a multicloning site, a T5NT sequence and the β-galactosidase reporter gene between two repetitions of the left TK flanking arm (Gómez *et al.*, 2007a, b). GFP expression was used as a readout in order to evaluate the promoter strength. For this purpose, the GFP gene was inserted into the pLZAW1 vector under the transcriptional control of the LEO (pLZAW1–LEO) or pS (pLZAW1–pS) promoter. These transfer vectors were used to generate the recombinant viruses MVA–LEO–GFP and MVA–PS–GFP following standard procedures described previously (Gómez *et al.*, 2007b). The purity of the recombinant viruses was assessed by PCR amplification of the insert in the TK locus. The viruses were grown in primary chicken embryo fibroblasts, purified through two 36 % (w/v) sucrose cushions and titrated by immunostaining assay as described previously (Ramírez *et al.*, 2000).

To define the temporal expression of GFP under the control of the two different promoters, non-permissive HeLa cells were infected at an m.o.i. of 5 with MVA–LEO–GFP or MVA–PS–GFP recombinant viruses. In the first 4 h of infection, cells infected with MVA–LEO–GFP expressed higher levels of GFP compared with cells infected with MVA–PS–GFP as determined by flow cytometry (Fig. 2a) and measured as mean fluorescence intensity (MFI) (Fig. 2b). At 8 h post-infection, no differences in the MFI between both viruses were observed, underlining that the strength of the late part of both promoters equals the fluorescence levels. These results were confirmed in a permissive chicken DF-1 cell line (data not shown).

To check if higher levels of antigen expression at early times post-infection correlated with an enhancement of the antigen-specific T-cell immune responses, we performed an immunization protocol using MVA–LEO–GFP and MVA–PS–GFP. BALB/c mice ($n=8$ per group) were injected by the intraperitoneal route with two doses of 1×10^7 p.f.u. of each virus with an interval of 1 week between the two inoculations. As a negative control, we used the parental virus MVA–WT. At 11 and 53 days after the last immunization, mice were sacrificed and spleens were processed for intracellular cytokine staining assay to study CD8 T-cell primary and memory immune responses, respectively. The splenocytes were stimulated for 6 h with 10 μg ml^{-1} of the GFP peptide HYLSTQSAL ($GFP_{200–208}$) (Centro Nacional de Biotecnología Proteomics Facility, Spain), which is able to induce a specific CD8 T-cell response, as reported previously (Gambotto *et al.*, 2000). The functional profile of the vaccine-induced T-cell response was determined by measuring the intracellular expression of the cytokines IFN-γ, IL-2 and TNF-α, as well as the exposure of lysosomal-associated membrane protein-1 (LAMP-1 or CD107a) on the surface of activated antigen-specific CD8 T-cells as a surrogate marker for induction of killing.

The magnitude of the CD8 T-cell response was defined as the number of CD8 T-cells expressing IL-2 and/or IFN-γ and/or CD107a and/or TNF-α, and the polyfunctionality as the capacity of CD8 T-cells to express more than one of these four activation markers. In primary and memory immune responses, the magnitude and polyfunctionality of the vaccine-induced GFP-specific CD8 T-cell immune response were significantly higher in mice infected with MVA–LEO–GFP than in those infected with MVA–PS–GFP (Fig. 3). In primary immune responses, the absolute frequencies of quadruple, triple, double and single GFP-specific CD8 T-cell populations were significantly higher in mice infected with MVA–LEO–GFP compared with MVA–PS–GFP-infected mice (Fig. 3c), whereas in memory immune responses only

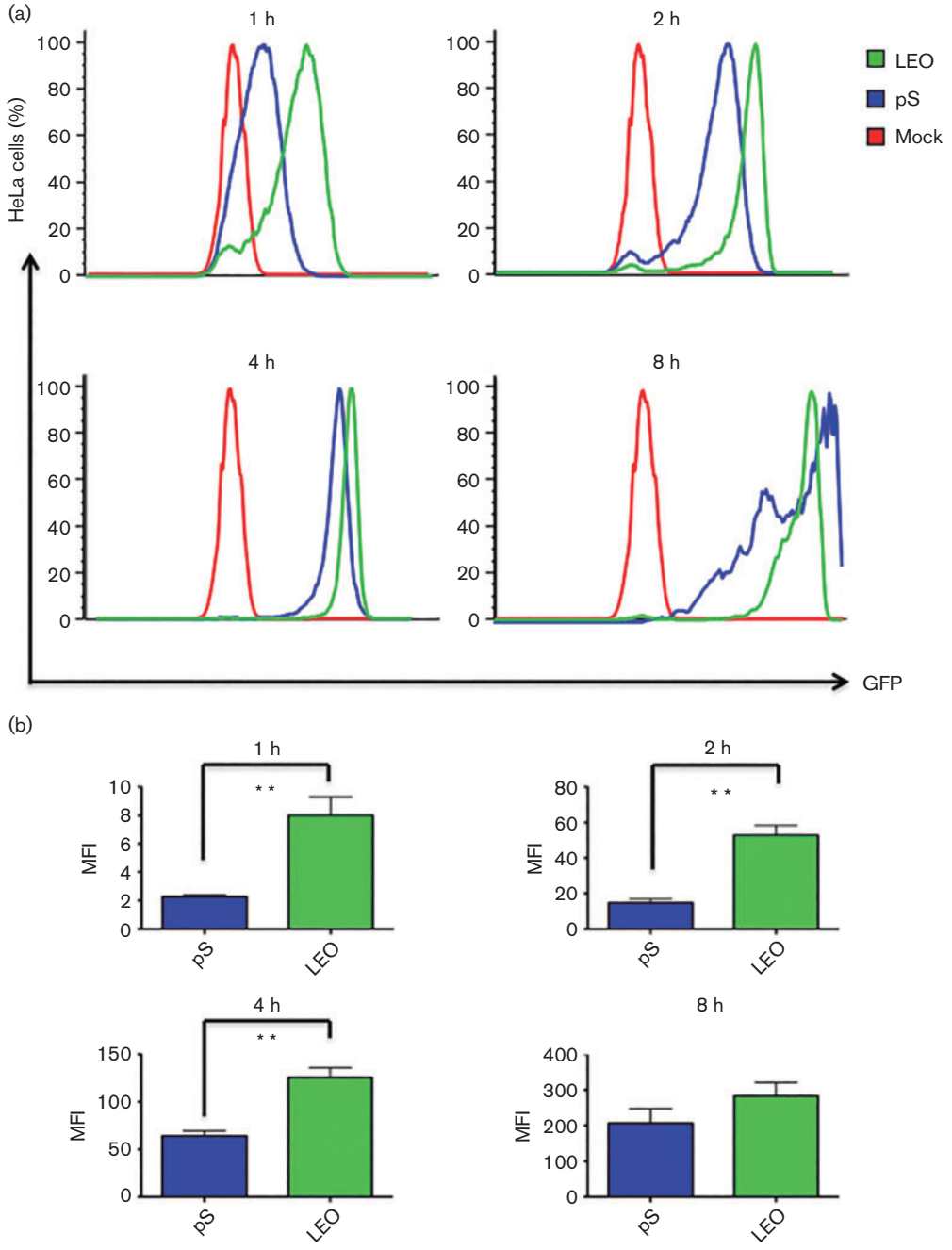

Fig. 2. GFP expression levels driven by LEO or pS promoters. (a) Expression of GFP in HeLa cells infected with MVA–LEO–GFP (LEO) or MVA–PS–GFP (pS) viruses at an m.o.i. of 5 at 1, 2, 4 and 8 h post-inoculation. (b) Mean fluorescence intensity (MFI) value of GFP expression compared with mock cells. The values represent the mean of three different experiments. $^{**}P<0.01$.

the quadruple CD8 T-cell subset was increased significantly (Fig. 3d). These results demonstrated that the enhanced immediate expression of the heterologous GFP antigen increased the quality of the antigen-specific T-cell response.

The LEO promoter represents the first example of a VACV synthetic promoter designed after bioinformatics analysis that shows an enhanced antigen expression within the first hour after infection and, importantly, is able to generate a significant increase in the antigen-specific CD8 T-cell immune response compared with that of the commonly used pS. This novel promoter represents an excellent prototype for future use to potentiate the expression of

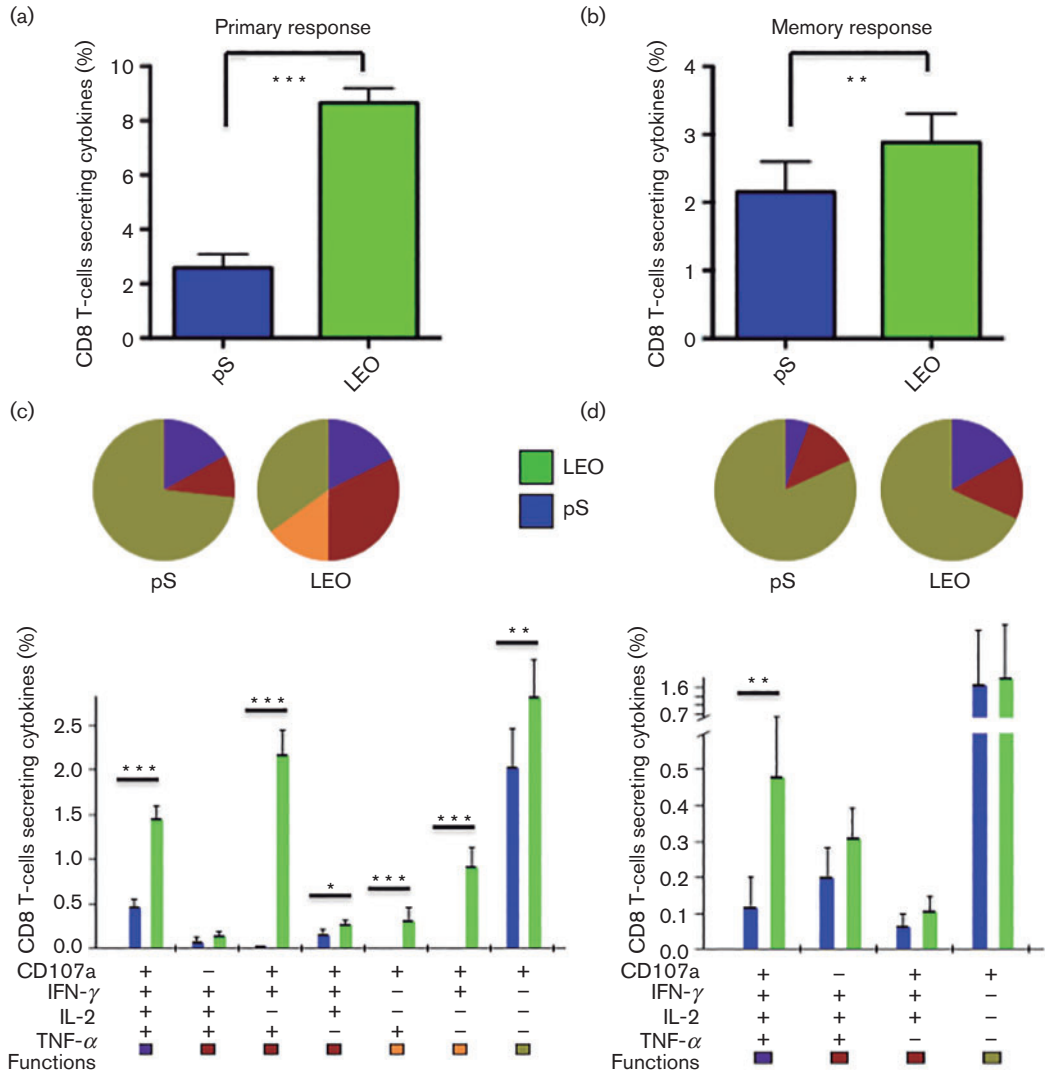

Fig. 3. Vaccine-induced GFP-specific CD8 T-cell immune responses in mice infected with MVA–PS–GFP (pS) or MVA–LEO–GFP (LEO) viruses. (a, b) Magnitude of the CD8 positive T-cell primary (a) and memory (b) immune responses of pooled splenocytes. The total value represents the sum of the percentages of CD8-positive T-cells secreting IL-2 and/or IFN-γ and/or CD107a and/or TNF-α. (c, d) Functional profile of primary (c) and memory (d) GFP-specific CD8 T-cells. The combinations of the responses are shown on the *x*-axis and the percentages of the functionally distinct populations are shown on the *y*-axis. The combinations with percentages <0.1 are not shown. Responses are grouped and colour coded on the basis of the number of functions. The non-specific responses obtained in mice infected with the control MVA–WT were subtracted in the total magnitude and in all combinations of CD8 response. The bars of the columns represent the confidence intervals of one experiment repeated twice. The statistical analysis used has been reported previously (García-Arriaza *et al.*, 2010). ***$P<0.001$, **$P<0.01$, *$P<0.05$.

antigens from different pathogens and to generate safe VACV recombinant-based vaccines able to induce potent immune responses that prevent development of the disease.

Acknowledgements

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3.3 Summary

3.3.1 Modification of promoter spacer length in vaccinia virus as a strategy to control the antigen expression

Several new early VACV promoters have been tested with different heterologous antigens and demonstrated to be more effective compared to the widely used pS synthetic promoter. These promoters differ in the spacer lengths between the gene and the early promoter sequence. It was nonetheless unknown how the length of the spacer between the early promoter motif and the gene under promoter control affect early antigen expression. To respond to this question, we generated several MVA vectors that express GFP under the transcriptional control of the synthetic late-early optimized (LEO) VACV promoter, using different spacer lengths. LEO promoter with a spacer length of 160 nucleotides (nt) increased significantly the GFP early expression compared to that of 99 and of 38 nt spacers.

In human and in murine models of *Leishmania major* infection, polyfunctional Th1 CD4 T cells correlated with vaccine-mediated protection. It was nevertheless unclear whether inducing heterologous early antigen expression can potentiate antigen-specific CD4 T cell responses. To answer this question, we generated two MVA vectors that express the LACK antigen (*Leishmania* homologue of receptors for activated C-kinase) under the transcriptional control of the LEO and LEO160 promoters. We demonstrated that LEO160 significantly enhanced LACK antigen expression compared to the LEO promoter. We showed that enhancement of *Leishmania* LACK early expression increased LACK-specific CD4 and CD8 T memory cell responses. Based on these inherent properties, we demonstrate that MVA-LEO160 is a prototype for the design of new poxvirus-based vaccine vectors.

Modification of promoter spacer length in vaccinia virus as a strategy to control the antigen expression

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Running title: Role of promoter spacer in poxvirus antigen expression
Keywords: Promoter; Spacer; Vaccinia virus; MVA; Vaccine; CD4 and CD8 T cell responses

Vaccinia viruses (VACV) with distinct early promoters have been developed to enhance antigen expression and improve antigen-specific CD8 T cell responses. It has not been demonstrated how the length of the spacer between a gene and its early promoter motif influences antigen expression, and whether the timing of gene expression can modify the antigen-specific CD4 T cell response. We generated several recombinant VACV based on the attenuated modified vaccinia Ankara (MVA) strain, which express GFP or the *Leishmania* LACK antigen under the control of an optimized promoter, using different spacer lengths. Longer spacer length increased GFP and LACK early expression, which correlated with an enhanced LACK-specific memory CD4 and CD8 T cell response. These results show the importance of promoter spacer length for early antigen expression by VACV and provide alternative strategies for the design of poxvirus-based vaccines.

INTRODUCTION

Poxvirus-based vaccines are used to generate strong antigen-specific T cell responses that control and clear pathogen infections (Ha et al., 2008). Poxvirus vectors are thus being tested as candidate vaccines to express heterologous pathogen antigens, and are being further improved to potentiate antigen-specific immune responses (immunogenicity) (Garcia-Arriaza and Esteban, 2014).

During a poxvirus infection, T cell immunogenicity correlates with antigen expression (Wyatt et al., 2008). The quantity of antigen production can affect the T cell immunodominance hierarchy (Wilson and Hunter, 2008), that is, the distinct levels of immunogenicity of all antigens expressed by a virus (Yewdell and Bennink, 1999). Recent studies of vaccinia virus (VACV) show that the efficiency with which an antigen is processed and presented on the surface of infected cells influences its recognition by immune system cells (Moutaftsi et al., 2009); 90% of all antigens recognized by CD8 T cells rank in the top 50% in terms of mRNA temporal expression (Sette et al., 2009). For this reason, efforts to develop new poxvirus vaccine candidates focus on using promoters to improve the timing of antigen expression and thus increase immune responses.

Vaccinia gene expression is controlled by early, intermediate and late promoters (Yang et al., 2013; Yang et al., 2011b). In mice, the timing of viral antigen expression correlates with better antigen-specific CD8 T cell responses (Bronte et al., 1997); early antigen-specific T cells have a proliferative advantage over late antigen-specific T cells (Kastenmuller et al., 2007). Early VACV promoters such as pHyB (Baur et al., 2010) psFJ1-10 (Isshiki et al., 2014; Sato et al., 2013) and PrMVA13.5-long (Wennier et al., 2013), which bear different tandem early promoter elements, have been tested with different heterologous antigens; they were shown to be more effective compared to the widely-used p7.5 and pS promoters (Chakrabarti et al., 1997; Cochran et al., 1985). A new LEO (late-early optimized) promoter, with an optimization of the early promoter motif was also compared to pS and used to increase heterologous early antigen expression and antigen-specific CD8 T cell responses (Di Pilato et al., 2013). These promoters differ in their early motif sequence and in spacer lengths between the gene and the early promoter. It is nonetheless not known how the length of the spacer between the early promoter motif and the gene under promoter control affects the early antigen expression.

CD4 T cells recognize VACV intermediate antigens better than early or late gene products (Yang et al., 2011b). CD4 T cells have a crucial role in mediating protection against a variety of pathogens such as human immunodeficiency virus (HIV), influenza, *Plasmodium falciparum* and *Leishmania* (Darrah et al., 2007; Reece et al., 2004; Roman et al., 2002; Younes et al., 2003). In man and in mouse models of *Leishmania major* infection, multifunctional T helper type 1 (Th1) CD4 T cells correlate with vaccine-mediated protection (Darrah et al., 2010; Macedo et al., 2012). It is nevertheless unclear whether it is possible to potentiate antigen-specific CD4 T cell responses by inducing heterologous early antigen expression during vaccinia virus infection. Modified vaccinia virus (MVA), a highly attenuated VACV, expressing LACK antigen (*Leishmania* homologue of receptors for activated C kinase), has been used as a safe, efficient vector able to induce a multifunctional, long-term, LACK-specific CD4 T cell response (Sanchez-Sampedro et al., 2012).

We generated several MVA vectors that express GFP or LACK antigens under the transcriptional control of the LEO promoter, with different spacer lengths. LEO promoter with a spacer length of 160 nucleotides (nt) increased GFP or LACK early expression compared to that of 38 nt. We show that enhancement of LACK early expression correlated with the length of the spacer, leading to increased LACK-specific CD4 and CD8 T memory cell responses.

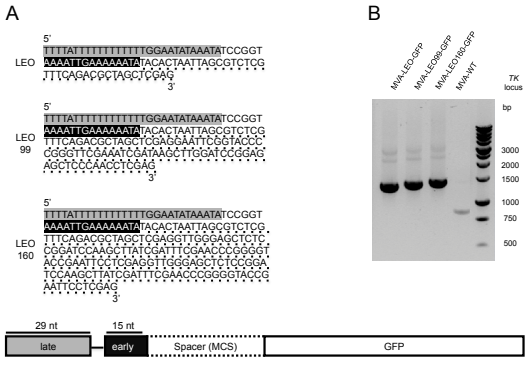

Figure 1. **Scheme and insertion of LEO, LEO99 and LEO160 promoters with GFP gene into MVA virus genome.** (A) Sequence of LEO, LEO99 and LEO160 promoters and scheme with the *GFP* gene. Late promoter element, grey box; early promoter motif, black box; spacer, white box with dashed line. (B) Promoter and *GFP* gene insertion into the MVA virus TK locus confirmed by PCR analysis. An 873 bp product was obtained in parental MVA, whereas in MVA-LEO-GFP, MVA-LEO99-GFP and MVA-LEO160-GFP recombinant viruses we observed 1312, 1373 and 1434 bp products, respectively.

RESULTS

Promoter spacer length and GFP antigen expression

We constructed LEO promoters with different spacer lengths. The LEO promoter has 29 nucleotides (nt) of the late element, 15 nt of the early motif and a 38 nt MCS spacer (Di Pilato et al., 2013). We designed two new promoters, LEO99 and LEO160 with the same late-early LEO composition and two MCS spacers of 99 or 160 nt Figure 1(A). The GFP open reading frame (ORF) was cloned downstream of LEO99 and LEO160 promoters into the VACV insertional pLZAW1 plasmid vector (Gomez et al., 2007a; Gomez et al., 2007b) and introduced into the MVA thymidine kinase (TK) locus (Gomez et al., 2007a; Gomez et al., 2007b). The MVA-LEO99-GFP and MVA-LEO160-GFP viruses were selected from MVA-WT by consecutive purification rounds (Gomez et al., 2007b). Absence of MVA-WT contamination and correct GFP insertion in the TK locus were confirmed by PCR amplification Figure 1(B).

Early GFP expression by MVA-LEO99-GFP and MVA-LEO160-GFP viruses was compared with that of the MVA-LEO-GFP virus, which induces significantly more early GFP expression than MVA-pS-GFP (Di Pilato et al., 2013). We infected human HeLa cells with the distinct MVA-GFP viruses at a multiplicity of infection (MOI) of 10 plaque-forming units (PFU) per cell and evaluated GFP expression in a time-course assay. In the first 6 h of infection, MVA-LEO160-GFP induced higher GFP levels compared to MVA-LEO99-GFP and MVA-LEO-GFP virus, as determined by flow cytometry Figure 2(A). To analyze early gene expression specifically, and to avoid contamination by late gene expression, the HeLa cells were treated for 6 h with cytosine arabinoside (AraC), an inhibitor of DNA replication. In these conditions of early phase MVA infection cycle arrest, MVA-LEO160-GFP induced more GFP expression than the other viruses Figure 2(A). Median fluorescence intensity (MFI) analysis of HeLa GFP levels showed that in the first 6 h of infection, alone or with AraC, MVA-LEO160-GFP significantly increased HeLa GFP production compared to MVA-LEO99-GFP or MVA-LEO-GFP Figure 2(B). The difference in GFP expression between MVA-LEO160-GFP and MVA-LEO-GFP was approximately two-fold from 2 to 6h post-infection. During the same period, MVA-LEO99-GFP induced significantly more GFP expression than MVA-LEO-GFP, indicating a correlation between spacer length and gene expression Figure 2(B).

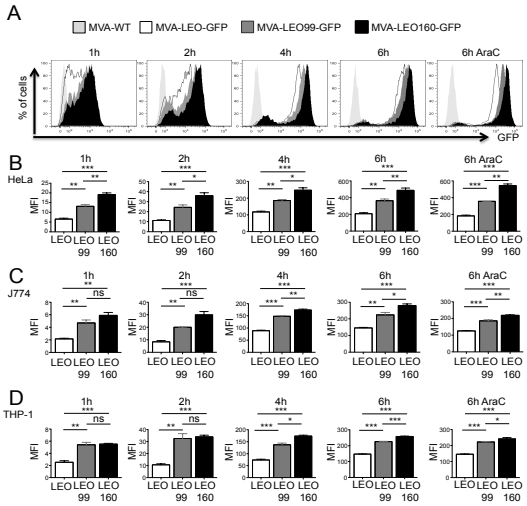

Figure 2. *In vitro* GFP expression driven by LEO, LEO99 and LEO160 promoters. (A) GFP expression in HeLa cells infected with MVA-WT, MVA-LEO-GFP, MVA-LEO99-GFP and MVA-LEO160-GFP viruses (MOI of 10 PFU/cell) at 1, 2, 4, 6 h post-infection, and with AraC (40 μg ml^{-1}) at 6 h post-infection. Graphs show *x*-fold increase in GFP median fluorescence intensity (MFI) of MVA-GFP compared to MVA-WT viruses in HeLa (B), J774 (C) and THP-1 cells (D). Note *y* axis scale difference for each condition. One representative example is shown of two independent experiments assayed in triplicate. Values shown as mean ± SEM. * p<0.05, ** p<0.01, *** p<0.001.

Results were similar for MVA-GFP viruses following infection of murine J774 macrophages and human THP-1 monocytes differentiated into macrophages Figure 2(C), (D). MVA-LEO160-GFP and MVA-LEO99-GFP significantly increased GFP levels compared to those induced by MVA-LEO-GFP; the difference between MVA-LEO160-GFP and MVA-LEO99-GFP was statistically significant from 4 h post-infection Figure 2(C), (D).

To determine whether infection with MVA-LEO160-GFP increased GFP expression *in vivo*, we infected BALB/c mice by i.p. injection of 10^7 PFU MVA-LEO-GFP or MVA-LEO160-GFP. Since macrophages are one of the most susceptible cell types to MVA infection (Brandler et al., 2010) and constitute ~30% of the total peritoneal cell yield (Ray and Dittel, 2010), only GFP^+ $F4/80^{high}$ macrophages were analyzed Figure 3(A); both viruses showed similar infective capacity (>80%) Figure 3(B). GFP^+ macrophages from MVA-LEO160-GFP-infected mice had significantly higher GFP levels than those from MVA-LEO-GFP-infected mice at 4 h post-infection Figure 3(C).

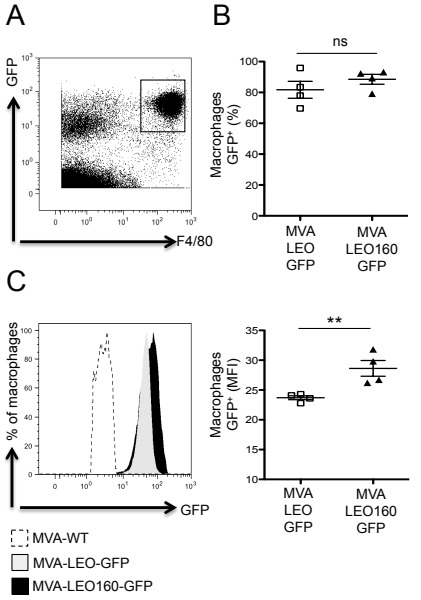

Figure 3. *In vivo* macrophage GFP expression driven by LEO and LEO160 promoters. (A) FACS plot (F4/80 macrophage marker vs. GFP) of peritoneal cells from MVA-LEO160-GFP-injected mice (10^7 PFU). (B) Percentage of GFP^+ macrophages from the peritoneal cavity of MVA-LEO-GFP- or MVA-LEO160-GFP-injected (10^7 PFU) mice at 4 h post-infection. (C) Expression (left panel) and median fluorescence intensity (MFI; right panel) of peritoneal GFP^+ macrophages at 4 h post-infection in MVA-LEO-GFP- or MVA-LEO160-GFP-injected mice. Graph shows *x*-fold increase in these macrophages compared to MVA-WT-injected mice. Values are mean ± SEM; each point represents an individual mouse. Data representative of two independent experiments. ** $p<0.01$.

Promoter spacer length and LACK antigen expression

Considering the strength of LEO160 promoter in inducing better early antigen expression among the promoters studied *in vitro*, we decided to compare its capacity to generate antigen-specific CD8 and CD4 T cell responses with the LEO promoter; we cloned the *Leishmania* LACK ORF downstream of LEO or LEO160 promoters Figure 4(A). To demonstrate that differences in early antigen expression were spacer length-dependent and not spacer sequence-dependent, we modified the spacer MCS sequence and maintained the same nt number in MVA-LEO160-LACK. The new VACV insertional pLZAW1 plasmid vectors were used to generate the MVA-LEO-LACK and MVA-LEO160-LACK viruses, which were selected from MVA-WT by consecutive purification rounds (Gomez et al., 2007b). Absence of MVA-WT contamination and correct LACK insertion in the TK locus were confirmed by PCR amplification Figure 4(B).

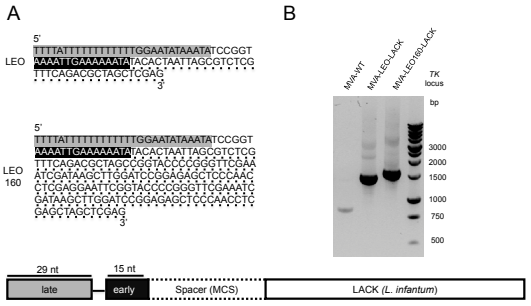

Figure 4. Scheme and insertion of LEO and LEO160 promoters with the LACK gene into MVA virus genome. (A) Sequences of LEO and LEO160 promoters and scheme with LACK gene. Late promoter element, grey box; early promoter motif, black box; spacer, white box with dashed line. (B) Promoter and LACK gene insertion into the MVA virus TK locus analyzed by PCR. An 873 bp product was obtained in parental MVA, whereas in MVA-LEO-LACK and MVA-LEO160-LACK recombinant viruses we observed 1486 and 1608 bp products, respectively.

The difference in LACK levels between these two viruses was tested by Western blot Figure 5(A) in HeLa cells infected at a MOI of 10 PFU/cell. From 2 to 6 h post-infection, alone or with AraC, MVA-LEO160-LACK significantly increased LACK expression (approximately two-fold) compared to MVA-LEO-LACK Figure 5(B), which confirmed the results obtained for the GFP antigen. LACK levels were normalized to E3 (VACV constitutive early protein) levels to show that the difference in heterologous antigen expression was the result of distinct promoter strength and not of different virus infective capacity. We found no differences in E3 expression between the viruses Figure 5(A). These *in vitro* and *in vivo* results indicate that promoter spacer length positively influences gene early expression under its control.

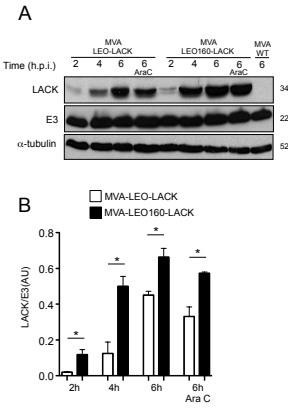

Figure 5. *In vitro* LACK expression driven by LEO and LEO160 promoters. (A) LACK expression in HeLa cells infected with MVA-WT, MVA-LEO-LACK or MVA-LEO160-LACK viruses (MOI of 10 PFU/cell) at 2, 4, 6 h and with AraC at 6 h post-infection. a-tubulin was used as a protein loading control. (B) Bars show the ratio of LACK to E3 bands, quantified using ImageJ software. Arbitrary unit (AU) values show the mean ± SEM of two independent experiments. * $p<0.05$.

LACK antigen-specific memory T cell responses

To determine whether increased LACK early expression enhances LACK-specific T cell responses, we infected BALB/c mice i.p. with two doses of MVA-LEO-LACK or MVA-LEO160-LACK virus, spaced by one week to avoid a high anti-vaccinia IgG antibody response (Hagensee et al., 1995) that could impair the MVA infective capacities. This homologous immunization protocol, in which mice were virus-inoculated more than once, was used because it is reported that differential antigen presentation changes the T cell immunodominance hierarchy between primary and secondary immunization (Crowe et al., 2003) and that this hierarchy correlates with viral gene expression after secondary immunization (Kastenmuller et al., 2007).

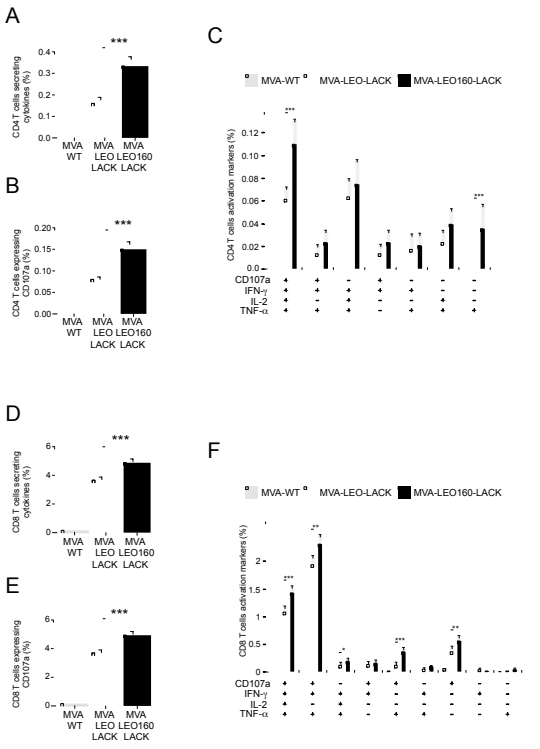

Figure 6. Vaccine-induced LACK-specific CD4 T and CD8 T memory cell responses in MVA-WT-, MVA-LEO-LACK- or MVA-LEO160-LACK-infected mice. The response was measured 66 days after the last immunization in mice (n = 4 per group). (A) Percentage of LACK-specific CD4 T cells secreting cytokines after two virus doses (10^7 PFU/mouse) spaced by one week. Values are the sum of the percentages of IL-2- and/or IFN-g- and/or TNF-a-secreting CD4 T cells. (B) Percentage of LACK-specific CD4 T cells expressing CD107a. (C) Functional profile of LACK-specific CD4 T memory cells. Combinations are shown of the responses (x axis) and percentages of functionally distinct populations (y axis). (D) Percentage of LACK-specific CD8 T cells secreting cytokines. Values are the sum of the percentages of IL-2- and/or IFN-g- and/or TNF-a-secreting CD8 T cells. (E) Percentage of LACK-specific CD8 T cells expressing CD107a. (F) Functional profile of LACK-specific CD8 T memory cells. Combinations are shown of the responses (x axis) and percentages of functionally distinct populations (y axis). Values shown as mean ± CI. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Since memory T cell responses are essential for inducing long-term protection against pathogen infection (Darrah et al., 2007; Reyes-Sandoval et al., 2011; Schmidt et al., 2008), at 66 days after the final virus dose, we measured the splenocyte T cell memory profile by intracellular cytokine staining (ICS). Splenocytes from infected mice were stimulated with purified LACK protein to study LACK-specific CD4 T cell responses, or with A20 cells previously nucleofected with the mammalian expression plasmid pcDNA-LACK to study LACK-specific CD8 T cell responses. To determine the functional profile of T cells, we measured levels of lysosomal-associated membrane protein-1 (LAMP-1/CD107a) as a surrogate marker for induction of killing, as well as gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α) and interleukin-2 (IL-2) levels. We defined the magnitude of the response as LACK-specific T cells that expressed CD107a or the sum of the frequencies of LACK-specific T cells secreting IFN-γ and/or TNF-α and/or IL-2. Polyfunctionality, defined as T cell capacity to express more than one of these four activation markers, determined the quality of the T memory cell response.

MVA-LEO160-LACK induced significantly more LACK-specific IFN-γ and/or TNF-α and/or IL-2-secreting CD4 T cells than MVA-LEO-LACK, when splenocytes were stimulated with LACK protein Figure 6(A). MVA-LEO160-LACK also generated significantly more LACK-specific CD107a-expressing CD4 T cells than MVA-LEO-LACK in virus-infected mice Figure 6(B). The quality of the LACK-specific CD4 T cell response showed that compared to MVA-LEO-LACK, MVA-LEO160-LACK induced a significant increase in quadruple activation marker-positive CD4 T cells Figure 6(C). The high polyfunctional profile generated was also represented by IFN-γ, TNF-α and IL-2 triple-positive CD4 T cells Figure 6(C); TNF-α single-positive CD4 T cells were the third most representative group.

The LACK-specific CD8 T cell profile showed that MVA-LEO160-LACK induced a significantly higher IFN-γ and/or TNF-α and/or IL-2^+ CD8 T cell response to LACK antigen Figure 6(D) compared to MVA-LEO-LACK; this significance difference was also detected in LACK-specific CD107a^+ CD8 T cells between two groups Figure 6(E). The polyfunctional CD8 T cell profile showed a clear predominance of the IFN-γ^+TNF-α^+CD107a^+ subset, followed by the quadruple marker-positive population Figure 6F). All of these data indicate that increased LACK-specific CD4 and CD8 T cell response induced by MVA-LEO160-LACK correlates with the enhanced LACK antigen expression.

DISCUSSION

Two categories of VACV early genes have been defined based on their temporal expression (Assarsson et al., 2008; Yang et al., 2010); their

promoter motif (consensus sequence) and their promoter spacers were also recently reported (Yang et al., 2011a). To define the role of the promoter spacer in the optimization of early antigen expression, we designed two new promoters, LEO99 and LEO160, with distinct spacer lengths and compared them with the previously reported LEO promoter (Di Pilato et al., 2013).

Compared to LEO, the LEO99 and LEO160 promoters (with 99 and 160 nt spacers) increased early antigen expression. The 50-nt window, from -70 nt to -20 nt before the ORF, is the most frequent site for the promoter motif of early genes and was used to generate the LEO promoter with a 38-nt spacer (Di Pilato et al., 2013). This result suggests that VACV, whose early genes rarely have spacers longer than 70 nucleotides, does not alone induce the highest possible gene expression. The DNA footprint of vaccinia RNA polymerase ternary complexes is less than 50 nt (Hagler and Shuman, 1992). The vaccinia virus early transcription factor (ETF) (Broyles et al., 1988) and RNA polymerase-associated protein (RAP94) (Ahn et al., 1994) mediate the promoter-RNA polymerase link. We hypothesized that spacers >50 nt would offer greater space to the transcription machinery, possibly accelerating gene expression, and defined here that spacers with more than 99 nt offer advantages of early gene expression. These new long spacers are maintained in the virus during multiple passages in cells, indicating stability within the virus genome.

Attenuated VACV vectors (MVA, ALVAC and NYVAC poxvirus strains) are used as candidate vaccines for emerging infectious diseases and cancer (Gomez et al., 2011). Several strategies have been developed to improve immunogenicity to heterologous antigens expressed by these vectors, including use of costimulatory molecules, administration of heterologous vectors, deletion of immunomodulatory viral genes and insertion of host range viral genes (Garcia-Arriaza and Esteban, 2014; Gomez et al., 2012, 2013). Early gene expression mediated by promoter optimization is another strategy to increase immunogenicity of the foreign antigen (Baur et al., 2010). Although it is reported that VACV intermediate antigens are the preferentially recognized by CD4 T cells (Yang et al., 2011b), we demonstrate that the early LACK expression driven by the early promoter of MVA-LEO160-LACK can positively influence LACK-specific CD4 T cell responses. While antigen-specific T cell responses can provide protective immunity against parasites (Belnoue et al., 2004), in a *Leishmania* infection model, only CD4 T cell immunogenicity correlated with this protection (Darrah et al., 2007).

Following *Leishmania major* infection, BALB/c mice show principally a Th2 response, due to the Th2 immunogenicity of the LACK antigen (Mougneau et al., 1995). As it is associated to greater susceptibility to *Leishmania* infection in BALB/c mice (Launois et al., 2007), a Th2 response must be avoided when using a LACK vaccine strategy in this strain. Conversely, the Th1 response protects against leishmaniasis infection (Darrah et al., 2010; Darrah et al., 2007). Th1 immune response was triggered by the MVA-LEO-LACK and MVA-LEO160-LACK, with clear differences in magnitude and polyfunctionality. We found that MVA-LEO160-LACK increased the number of IL-2^+IFNγ^+TNFα^+ CD4 Th1 cells. Compared to MVA-LEO-LACK, MVA-LEO160-LACK induced more antigen-specific CD4 T effector memory cells, which are necessary for protection against *Leishmania* reinfection (Peters et al., 2014). MVA-LEO160-LACK also enhanced CD107a$^+$ memory CD4 T cells to LACK. CD107a$^+$ CD4 T cells are resistant to HIV-1 infection, which implies that these cells could control the infection (Terahara et al., 2014). Compared to MVA-LEO-LACK, we also demonstrated that MVA-LEO160-LACK increased the magnitude of polyfunctional CD8 T cells that express IL-2 and IFNγ; these cell subsets have a role in maintaining Th1 polarization of CD4 T cells (Gurunathan et al., 2000), limiting the natural Th2 response (Uzonna et al., 2004).

Although the role of memory CD8 T cells against *Leishmania* remains to be defined, CD8 T memory cells have a clear protective function against parasites (Reyes-Sandoval et al., 2011; Rigato et al., 2011). The capacity of MVA-LEO160-LACK viral vector to increase CD8 and CD4 T memory cell responses against a heterologous antigen could be useful in other infection models where a robust presence of both T cell subtypes has been associated with protection and control of disease (Hansen et al., 2011; Hansen et al., 2009).

In summary, we have identified how VACV promoters with different spacer lengths can be used to enhance the levels of foreign antigens in infected cells. We defined *in vitro* and *in vivo* the strength of MVA-LEO160, as a valid strategy to induce early heterologous antigen expression. In mouse studies, the magnitude and the quality of the CD4 and CD8 T memory responses defined the improved immune properties of this vector. Based on its capacity to increase early antigen expression and antigen-specific CD4 and CD8 T cell responses, MVA-LEO160 is a prototype for the design of new poxvirus-based vaccine vectors.

METHODS

Mice and injections

BALB/c mice (6-8 weeks old) were purchased from Harlan (Interfauna Ibérica, Sant Feliu de Codines, Spain). For the homologous immunization protocol to assay T cell immunogenicity, mice received two intraperitoneal (i.p.) injections (one every 7 days) of 10^7 PFU of MVA-WT, MVA-LEO-LACK or MVA-LEO160-LACK. Animal studies were approved by the Ethical Committee of Animal Experimentation (CEEA-CNB) of the Centro Nacional de

Biotecnologia (CNB-CSIC, Madrid, Spain) in accordance with national and international guidelines and Royal Decree (RD 1201/2005) (permit nº 11037, 13012).

Cells
Human epithelial cervix adenocarcinoma cells (HeLa), immortalized chicken embryo fibroblast cells (DF-1; both from the American Type Culture Collection, Manassas, VA) and primary chicken embryo fibroblasts (CEF; Intervet, Salamanca, Spain) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FCS. Human monocytic THP-1 cells, murine macrophage J774.G8 cells and B lymphocytes derived from a reticulum cell sarcoma (A20; all from ATCC) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 50 μM 2-mercaptoethanol and 10% (v/v) FCS. THP-1 cells were differentiated into macrophages by treatment with 5 ng ml^{-1} phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 24 h before use. Cells were cultured in humidified air, 5% (v/v) CO_2 at 37°C.

Viruses
The recombinant MVA-GFP and MVA-LACK viruses were constructed using GFP as fluorescent marker and β-galactosidase as reporter gene, respectively. DF-1 cells were infected with 0.01 PFU/cell MVA-WT (1 h) and transfected with VACV insertional plasmid vectors pLZAW1-LEO (Di Pilato et al., 2013), pLZAW1-LEO99 or pLZAW1-LEO160 using Lipofectamine (Invitrogen). Recombinant viruses were selected from progeny virus by consecutive rounds of plaque purification, as reported (Gomez et al., 2007a; Gomez et al., 2007b). *In vitro* virus infections were performed in medium with 2% (v/v) FCS. All viruses were grown in primary CEF cells, similarly purified through two 36% (w/v) sucrose cushions, and titers were determined by immunostaining plaque assay in DF-1 cells, as described (Ramirez et al., 2000).

Plasmids
The plasmid transfer vectors pLZAW1-LEO99 and pLZAW1-LEO160 were obtained by inserting part of the pGEM-7Zf(-) (Promega) multi-cloning site (MCS) into the pLZAW1-LEO plasmid. The plasmid transfer vectors pLZAW1-LEO-GFP, pLZAW1-LEO99-GFP, pLZAW1-LEO160-GFP, pLZAW1-LEO-LACK and pLZAW1-LEO160-LACK were obtained by cloning the GFP or *Leishmania infantum* LACK sequences into the pLZAW1-LEO, pLZAW1-LEO99 or pLZAW1-LEO160 plasmids.

Protein
Leishmania infantum LACK protein was expressed as a fusion protein with an N-terminal histidine tag using the *E. coli* strain BL21 pLysS transformed with the pRSET-B-LACK plasmid (Gonzalez-Aseguinolaza et al., 1999), which allows affinity chromatography purification on a Ni^{2+} column. LACK expression was induced by addition of isopropyl thio-β-D-galactoside (37ºC).

PCR
Viral DNA was extracted by the SDS-proteinase K-method (Perdiguero et al., 2013). Primers TK-L and TK-R were used for PCR analysis of the *TK* locus, as described (Gomez et al., 2007a), to confirm GFP and LACK gene insertion. The primers GFP-Fwd CCGCTCGAGATGGCTAGCAA AGGAGAAGAAC and GFP-Rev AACTGCAGTTC TCAAGCTATGCATCCAAC, the primers LACK-Fwd CTAGCTAGCTCGAGATGAACTACGAGGG TCACC and LACK-Rev AACTGCAGTTACTCGG CGTCGGAGATGG, and the primers MCS-Fwd CCGCTCGAGGAATTCGGTACCCC and MCS-Rev CCGCTCGAGGTTGGGAGCT CTCC were used for GFP, LACK and MCS amplification, respectively.

Western blot
HeLa cells were lysed in Laemmli buffer, extracts fractionated by 8% (v/v) SDS-PAGE and analyzed by Western blot using rabbit polyclonal anti-LACK, -E3 (both from the Centro Nacional de Biotecnología) or -tubulin antibodies (Cell Signaling).

Nucleofection assay
A20 cells were nucleofected using 4D-Nucleofector (Lonza) and the Amaxa SF Cell Line kit (Lonza). Cells were nucleofected with 6 μg of pCIneo-LACK plasmid; pCIneo-ϕ was used as negative control. At 24 h post-nucleofection, A20 cells were used to restimulate splenocytes from infected mice (1:10 A20:splenocyte ratio).

Flow cytometry
For intracellular cytokine staining (ICS), erythrocyte-depleted splenocytes were rested overnight, resuspended in RPMI 1640 with 10% (v/v) FCS and 1 μg ml^{-1} Golgiplug (Becton Dickinson; BD), monensin (eBioscience) and anti-CD107a (1D4B; BD). After restimulation with 25 μg ml^{-1} LACK protein or A20 cells (6 h, 37°C), splenocytes were stained for surface markers using anti-CD3 (145-2C11), -CD4 (GK1.5), -CD8 (53-6.7), -CD62L (MEL-14) (all from BD) and -CD127 (A7R34) (eBioscience), then fixed, permeabilized (Cytofix/Cytoperm kit; BD), and stained intracellularly with anti-IL-2 (JES6-5H4), -IFN-γ (XMG 1.2) and -TNF-α (MP6-X722) (all from BD). Peritoneal exudate cells (PEC), obtained after injection of cold PBS into previously infected mice, were counted and stained with anti-F4/80 (BM8, eBioscience). Dead cells were stained using the violet LIVE/DEAD stain kit (Invitrogen) in all flow-cytometry analyses. Cells were acquired using a GALLIOS (Beckman Coulter) or LSRII (BD) flow cytometer; data analyses were performed with FlowJo software v. 8.5.3 (Tree Star). Boolean combinations of single functional gates were created with FlowJo to determine the frequency of each response based on all possible combinations of cytokines or of differentiation marker expression.

Statistical analysis
For statistical analysis of the T cell response to LACK antigen, we used an approach that corrects

measurements for medium response and allows calculation of confidence intervals (CI) and *p* values of hypothesis tests (Najera et al., 2010). Only antigen response values significantly larger than the corresponding RPMI condition are shown. Background values were subtracted from all values used to allow analysis of proportional representation of responses. Distribution was analyzed and presented using SPICE version 5.1, downloaded from http://exon.niaid.nih.gov. For statistical analysis of antigen expression, Student's *t*-test was applied to compare the viruses used.

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AUTHOR CONTRIBUTIONS

M.E. supervised the work; M.D.P designed research; M.D.P, L.S.-S. and E.M.-P-performed research; M.D.P contributed new reagents/analytic tools; M.D.P and C.O.S.S. analyzed data; and M.D.P wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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3.4 Summary

3.4.1 Deletion of the vaccinia virus gene *A46R*, encoding for an inhibitor of TLR signalling, is an effective approach to enhance the immunogenicity in mice of the HIV/AIDS vaccine candidate NYVAC-C

Distinct MVA (modified vaccinia virus Ankara) recombinants have been generated to target Toll-like receptor (TLR) pathways that, by inducing innate immune responses after pathway activation, are indirectly involved in the generation of antigen-specific T cell responses. By binding the Toll-like-interleukin-1 receptor adaptors and disrupting the interaction between TLR and these adaptors, vaccinia virus A46 protein inhibits activation of the interferon (IFN) type I signal. The role of A46 in the generation of immune responses to heterologous antigens has not been described.

To respond to this question, we generated NYVAC, a highly attenuated VACV strain that expresses HIV-1 clade C antigens but lacks the specific *A46R* viral gene. The NYVAC-C ΔA46R virus induced a higher pro-inflammatory response compared to NYVAC-C virus, as indicated by cytokine/chemokine levels in infected macrophages. In mice, the new deletion mutant increased the HIV-1 CD8 T cell memory and adaptive responses as well as the HIV-1 CD4 T cell adaptive response.

These results confirm the role of A46 in inducing pro-inflammatory signals, demonstrate the link between the TLR pathway and heterologous antigen-specific adaptive/memory responses, and lay a foundation for similar approaches in the design of vaccinia virus candidate vaccines.

PLOS ONE

Deletion of the Vaccinia Virus Gene *A46R*, Encoding for an Inhibitor of TLR Signalling, Is an Effective Approach to Enhance the Immunogenicity in Mice of the HIV/AIDS Vaccine Candidate NYVAC-C

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Abstract

Viruses have developed strategies to counteract signalling through Toll-like receptors (TLRs) that are involved in the detection of viruses and induction of proinflammatory cytokines and IFNs. Vaccinia virus (VACV) encodes A46 protein which disrupts TLR signalling by interfering with TLR: adaptor interactions. Since the innate immune response to viruses is critical to induce protective immunity, we studied whether deletion of *A46R* gene in a NYVAC vector expressing HIV-1 Env, Gag, Pol and Nef antigens (NYVAC-C) improves immune responses against HIV-1 antigens. This question was examined in human macrophages and in mice infected with a single *A46R* deletion mutant of the vaccine candidate NYVAC-C (NYVAC-C-ΔA46R). The viral gene *A46R* is not required for virus replication in primary chicken embryo fibroblast (CEF) cells and its deletion in NYVAC-C markedly increases TNF, IL-6 and IL-8 secretion by human macrophages. Analysis of the immune responses elicited in BALB/c mice after DNA prime/NYVAC boost immunization shows that deletion of *A46R* improves the magnitude of the HIV-1-specific CD4 and CD8 T cell immune responses during adaptive and memory phases, maintains the functional profile observed with the parental NYVAC-C and enhances anti-gp120 humoral response during the memory phase. These findings establish the immunological role of VACV *A46R* on innate immune responses of macrophages *in vitro* and antigen-specific T and B cell immune responses *in vivo* and suggest that deletion of viral inhibitors of TLR signalling is a useful approach for the improvement of poxvirus-based vaccine candidates.

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Introduction

The search for a safe and effective HIV vaccine able to elicit long-lasting protective immunity has encouraged the development of recombinant live vaccine candidates with good safety and immunogenicity profiles. The Thai phase III clinical trial (RV144) using the recombinant poxvirus vector ALVAC and the protein gp120 in a prime-boost strategy and showing a 31.2% protection against HIV infection [1], has raised considerable interest in the use of improved attenuated poxvirus recombinants as HIV vaccine candidates. Among poxviruses, the highly attenuated vaccinia virus (VACV) strain NYVAC is under intense preclinical and clinical evaluation as a vaccine against emergent infectious diseases and cancer [2].

The NYVAC strain was derived from a plaque clone isolate of the Copenhagen vaccinia virus strain (VACV-COP) by the deletion of 18 open reading frames (ORFs) involved in virulence, pathogenesis and host range functions [3]. In spite of its limited replication in human and most mammalian cell types, NYVAC provides a high level of gene expression and induces antigen-specific immune responses when administered to animals and humans [2,4,5,6]. However, the vector still

contains other immunomodulatory viral genes that may suppress host immunity, particularly genes encoding proteins that antagonize the innate immune response mediated by Toll-like receptor (TLR) signalling. The deletion of these immunomodulatory genes could be a strategy to further improve NYVAC-based vaccines with the aim to obtain enhanced magnitude, breadth, polyfunctionality and durability of the immune responses.

The sensing of viral pathogens and the subsequent innate immune responses triggered are critical to produce protective immunity. Cells of the innate immune system detect viruses through the recognition of specific pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) [7,8,9,10], among which TLRs are the best characterized [11]. TLR3, TLR7/8 and TLR9 reside predominantly within the endosomes where they recognize viral nucleic acids being involved in the generation of potent antiviral responses [12] while viral glycoprotein products have been shown to interact with TLR2 and TLR4 expressed on the cell surface [13,14]. The implication of TLR2 in the induction of type I IFN in inflammatory monocytes following *in vivo* infection with VACV has been reported and depletion of these cells leads to elevated levels of VACV in ovaries of mice [15]. TLR2 signalling has also been shown to be important for clonal expansion and memory CD8 T cells formation following VACV infection [16] and in VACV-induced production of proinflammatory cytokines by murine denditic cells (DCs) [17]. The best known role of TLR4 is the detection of lipopolysaccharide (LPS) but this receptor is also involved in the immune response to viruses. For example, TLR4 has been reported to be protective in pulmonary VACV infection since mice deficient for TLR4 signalling showed enhanced viral replication, hypothermia and mortality compared to control animals [18]. Because TLRs are expressed both on specific nonimmune cells, such as epithelial cells at potential sites of entry, and on a variety of immune cells including macrophages, DCs, B cells and certain types of T cells, they play a key role in the defence against pathogens through the induction of proinflammatory cytokines and type I IFNs but also in shaping pathogen-specific humoral and cellular adaptive immune responses.

All TLRs are type I transmembrane glycoprotein receptors comprised of an extracellular N-terminal leucine-rich repeat (LRR) domain involved in ligand binding, a single transmembrane domain and an intracellular C-terminal domain, known as the Toll/IL-1 receptor (TIR) domain, which mediates the interaction and recruitment of various adaptor proteins to activate the downstream signalling pathway [19]. PAMP binding induces receptor homo- or heterodimerization [20,21] and this activated conformation of the receptor triggers the recruitment of TIR domain-containing adaptor proteins that connect downstream signalling molecules leading to the activation of transcription factors such as IFN regulatory factors (IRFs) and NF-κB and the induction of type I IFNs and proinflammatory cytokines, respectively. Ligand recognition by TLRs induces the recruitment of five different adaptor proteins: Myeloid differentiation factor 88 (MyD88), MyD88-adaptor-like (Mal), TIR domain-containing adaptor protein-inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and sterile α- and armadillo-motif-containing protein (SARM) [22]. Two major pathways can be activated by TLRs: the MyD88-dependent pathway, used by all TLRs except TLR3 [23] and the TRIF-dependent pathway, used by TLR3 and TLR4. TLR4 is the only receptor being able to signal via both pathways due to the differential use of two adaptors, TRAM and Mal. TLR4 uses TRAM to recruit TRIF and induce a type I IFN response via the TRIF-dependent pathway while the use of the coadaptor Mal to recruit MyD88 via the MyD88-dependent pathway induce a proinflammatory response [24]. Crystal structures of the TIR domains of TLR2 [25], TLR10 [26], interleukin-1 receptor accessory protein-like (IL-1RAPL) [27] and Mal [28,29] and NMR structure of the TIR domain of MyD88 [30] have been determined. These studies identified a conserved protruding BB loop between the βB strand and the αB helix, which is essential for functional TLR signalling [31,32,33,34,35].

Viruses have developed strategies to target TLR-mediated signalling to manipulate and evade the host innate immune response [36]. VACV encodes some intracellular negative regulators of TLR signalling including A46 [37], A52 [38], N1 [39], B14 [40], K7 [41] and C6 [42]. A46 was the first virally encoded protein identified to contain a TIR domain [37,43]. Through this domain, A46 binds directly to the TIR domain-containing adaptors MyD88, Mal, TRIF and TRAM, disrupting the formation of Receptor: Adaptor TIR interactions [37] and therefore inhibiting downstream signalling to MAPKs, NF-κB and IRF-3 and interfering with both proinflammatory and type I IFN responses [37]. However, A46 does not interact with SARM, which is a negative regulator of TLR signalling [37]. It has also been shown that A46 protein contributes to virulence since VACV *A46R* deletion mutant was attenuated in a murine intranasal model [37]. An 11 amino acid peptide derived from A46 (called VIPER) has been reported to specifically inhibit TLR4 responses by directly targeting Mal and TRAM [44] and that A46 binds to Mal via a Bcl-2-like α-helical dimer subdomain [45]. The molecular basis for A46 antagonism of TLR4 has been recently reported [46]. A46 has been shown to impair TLR4 signalling by targeting the conserved BB loop of TIR proteins and thereby disrupting Receptor: Adaptor TIR interactions [46].

Since VACV has been reported to be sensed by TLR2 [15,16,17], TLR4 [18], TLR2-TLR6-MyD88, MDA-5/IPS-1 and NALP3 inflammasome [47] and *A46R* targets the TIR domain of the adaptors MyD88, Mal, TRIF and TRAM [37], in the present study we have asked to what extent *A46R* impacts on the immune responses against VACV. This question was addressed with NYCAC-C, an attenuated poxvirus vector expressing HIV-1 Env and Gag-Pol-Nef (GPN) antigens from clade C [48], where *A46R* was deleted (NYVAC-C-ΔA46R). Specific innate, adaptive and memory immune responses to HIV-1 antigens were evaluated in human macrophages and in a BALB/c mouse model comparing the recombinant virus in the presence or absence of *A46R*. Our findings provided evidence for an immunomodulatory role of VACV A46 protein.

Results

Generation and *in vitro* characterization of NYVAC-C-ΔA46R deletion mutant

NYVAC-C-ΔA46R deletion mutant was generated as detailed under Materials and Methods using as parental virus the recombinant NYVAC-C that expresses the HIV-1 Env, Gag, Pol and Nef antigens from clade C [48] and following a strategy that allows the deletion of the gene of interest with no fluorescent marker included in the final deletion mutant. The correct deletion of *A46R* gene was confirmed by PCR using primers annealing in *A46R* flanking sequences. As shown in Figure 1A, *A46R* ORF was successfully deleted and no wild-type contamination was present in NYVAC-C-ΔA46R preparation. Analysis by Western-blot confirmed that the *A46R* deletion mutant expresses the HIV-1 proteins gp120 and GPN at the same level as the parental virus NYVAC-C (Figure 1B). Moreover, analysis by immunostaining showed that all virus plaques have immunoreactivity to anti-WR, anti-gp120 and anti-gag p24 antibodies (data not shown), demonstrating the stability of the antigens expressed by the *A46R* deletion mutant. To determine if deletion of *A46R* gene affects virus replication, we compared the growth kinetic of NYVAC-C-ΔA46R deletion mutant with its parental virus NYVAC-C in CEF cells. Figure 1C shows that the growth kinetics were similar between parental and deletion mutant, indicating that *A46R* gene is not required for virus replication in cultured cells and its deletion does not affect virus growth kinetics.

NYVAC-C-ΔA46R up-regulates TNF, IL-6 and IL-8 production by human macrophages

To define whether *A46R* impairs the response of innate immune cells to NYVAC-C, we measured by ELISA the concentrations of proinflammatory cytokines and chemokines released by human THP-1 macrophages infected for 6 hours with 1 or 5 PFU/cell of NYVAC-WT, NYVAC-C or NYVAC-C-ΔA46R. Compared to NYVAC-WT and to NYVAC-C, the *A46R* deletion markedly up-regulated the production of TNF, IL-6 and IL-8 by THP-1 cells (Figure 2). Thus, the single deletion of *A46R* in the NYVAC-C genome triggers a stronger innate immune sensing than NYVAC-C, providing evidence for immune suppression by *A46R*.

Deletion of the viral gene *A46R* in NYVAC-C induces high, broad and polyfunctional HIV-1-specific T cell adaptive immune responses in BALB/c mice in heterologous prime/boost combination

To assay *in vivo* the effect of *A46R* gene deletion on the cellular immunogenicity against HIV-1 antigens, we analyzed the HIV-1-specific T cell adaptive immune responses elicited in mice by using a DNA prime/Poxvirus boost approach since it has been extensively reported that this heterologous immunization protocol is more immunogenic than either component alone to activate T cell responses to HIV-1 antigens [48,49,50].

BALB/c mice, 4 in each group, were immunized as described in Materials and Methods and adaptive T cell immune responses were measured 10 days after the last immunization by polychromatic intracellular cytokine staining (ICS) assay. Splenocytes from immunized animals were stimulated *ex vivo* for 6 hours with a panel of 464 peptides (15 mers overlapping by 11 amino acids) grouped in three pools: Env (112 peptides), Gag (121 peptides) and GPN (231 peptides) and stained with specific antibodies to identify T cell lineage (CD3, CD4 and CD8), degranulation (CD107a) and responding cells (IL-2, IFN-γ and TNF-α). The percentages of T cells producing IFN-γ and/or IL-2 and/or TNF-α established the overall $CD4^+$ T cell responses whereas the percentages of T cells producing CD107a and/or IFN-γ and/or IL-2 and/or TNF-α determined the overall $CD8^+$ T cell responses.

As shown in Figure 3A, in both immunization groups DNA-C/NYVAC-C and DNA-C/NYVAC-C-ΔA46R the magnitudes of the HIV-1-specific CD4 or CD8 T cell responses, determined as the sum of the individual responses obtained for Env, Gag and GPN peptide pools, were significantly higher than those obtained in the control group DNA-ϕ/NYVAC-WT ($p<0.001$). Furthermore, the magnitudes of the HIV-1-specific CD4 or CD8 T cell responses in the group immunized with NYVAC-C-ΔA46R were significantly higher than those obtained in the group DNA-C/NYVAC-C ($p<0.001$). In animals immunized with the parental NYVAC-C, the $CD4^+$ T cell response was only directed against the Env pool while in the group boosted with the NYVAC-C-ΔA46R deletion mutant this response was mainly mediated by Env pool but the response against Gag and GPN peptide pools also contributes to the total HIV-1-specific CD4 T cell response. On the other hand, the $CD8^+$ T cell responses were higher in magnitude and *A46R* gene deletion induced a significant enhancement in the magnitude of the $CD8^+$ T cell responses against the Env pool ($p<0.001$) whereas the anti-GPN response was maintained. Representative functional profiles of Env-specific CD4 or CD8 T cell responses are shown in Figure 3B.

The quality of a T cell response can be characterized in part by the pattern of cytokine production and by the cytotoxic potential. On the basis of the analysis of IFN-γ, IL-2 and TNF-α secretion, as well as the study of CD107a expression on the surface of activated T cells as an indirect marker of cytotoxicity, 8 HIV-specific CD4 T cell populations and 16 HIV-specific CD8 T cell populations were identified. Vaccine-induced CD4 T cell responses were highly polyfunctional in both DNA-C/NYVAC-C and DNA-C/NYVAC-C-ΔA46R groups, with more than 60% of CD4 T cells exhibiting two or three functions. CD4 T cells producing IFN-γ+IL-2+TNF-α, IL-2+TNF-α or only TNF-α or IL-2 were the most representative populations induced by the parental NYVAC-C and the *A46R* deletion mutant, although the percentages of cells producing cytokines were low (Figure 3C). The HIV-1-specific CD8 T cell responses, higher in magnitude, were also polyfunctional in both immunization groups, with more than 50% of $CD8^+$ T cells exhibiting two, three or four functions. $CD8^+$ T cells producing CD107a+ IFN-γ+TNF-α, CD107a+ TNF-α or only CD107a were the most representative populations induced by the parental NYVAC-C and NYVAC-C-ΔA46R deletion mutant (Figure 3C).

Overall, these results indicate that deletion of *A46R* gene from NYVAC-C genome improved the magnitude of the HIV-1-specific adaptive CD4 and CD8 T cell immune responses and maintained the polyfunctional profile observed with the parental

Figure 1

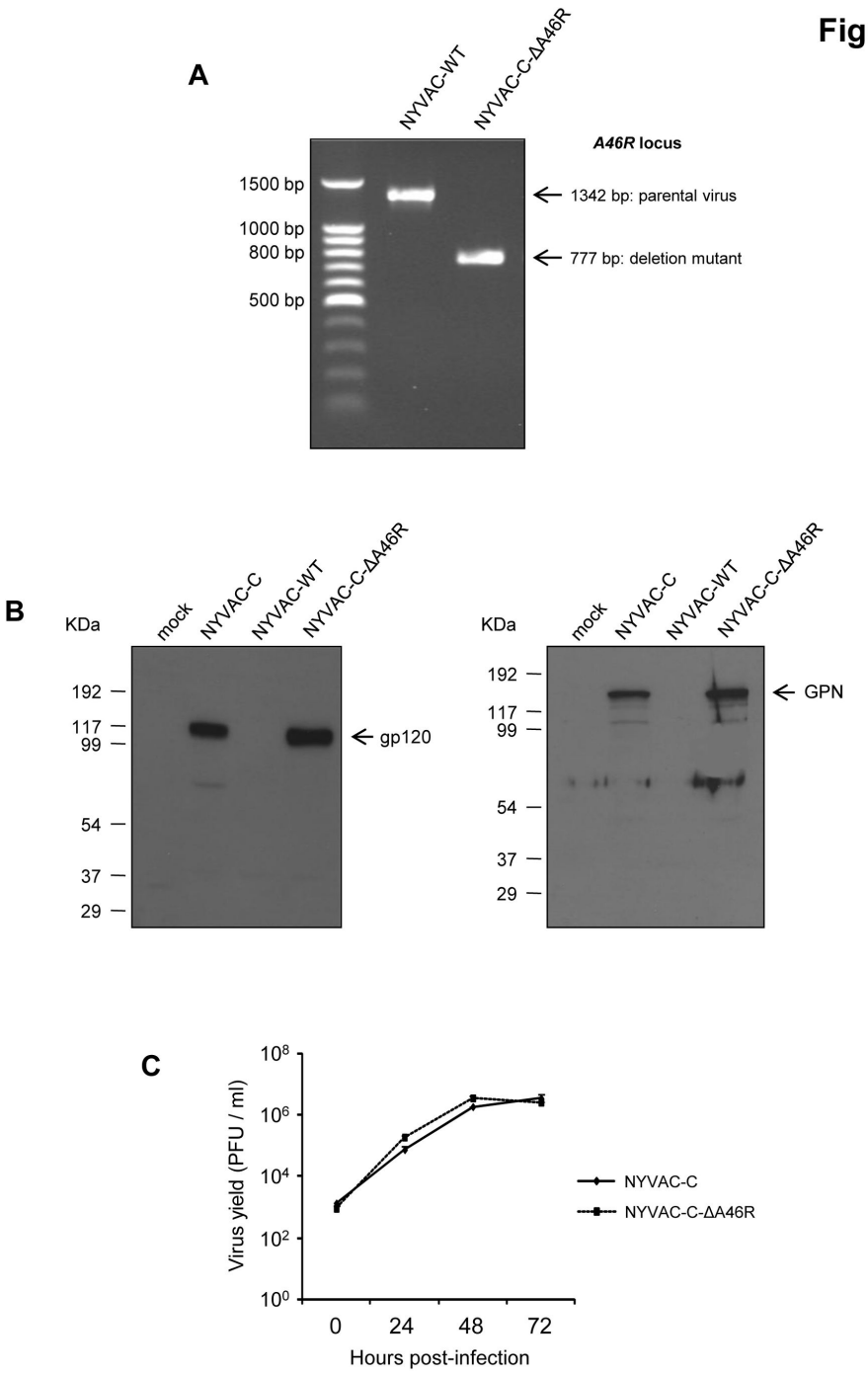

Figure 1. In vitro characterization of NYVAC-C-ΔA46R deletion mutant. (A) Confirmation of *A46R* gene deletion by PCR analysis. Viral DNA was extracted from BSC-40 cells infected with NYVAC-WT or NYVAC-C-ΔA46R at 5 PFU/cell. Primers LFA46R-Aat and RFA46R-Bam spanning *A46R* flanking sequences were used for PCR analysis of *A46R* locus. In parental NYVAC, a 1342 bp-product is obtained while in deletion mutant a unique 777 bp-product is observed. (B) Expression of HIV antigens by Western-blot. BSC-40 cells were mock-infected or infected at 5 PFU/cell with NYVAC-WT, NYVAC-C or NYVAC-C-ΔA46R. At 24 hours post-infection, cells were lysed in Laemmli buffer, cells extracts were fractionated by 8% SDS-PAGE and analyzed by Western-blot using a polyclonal anti-gp120 antibody or a polyclonal anti-gag p24 serum to evaluate the expression of gp120 and GPN proteins, respectively. (C) Analysis of virus growth of NYVAC-C-ΔA46R in CEF cells. Monolayers of CEF cells were infected with NYVAC-C or NYVAC-C-ΔA46R at 0.01 PFU/cell. At different times post-infection (0, 24, 48 and 72 hours), cells were collected and infectious viruses were quantified by immunostaining plaque assay in BSC-40 cells.

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Figure 2

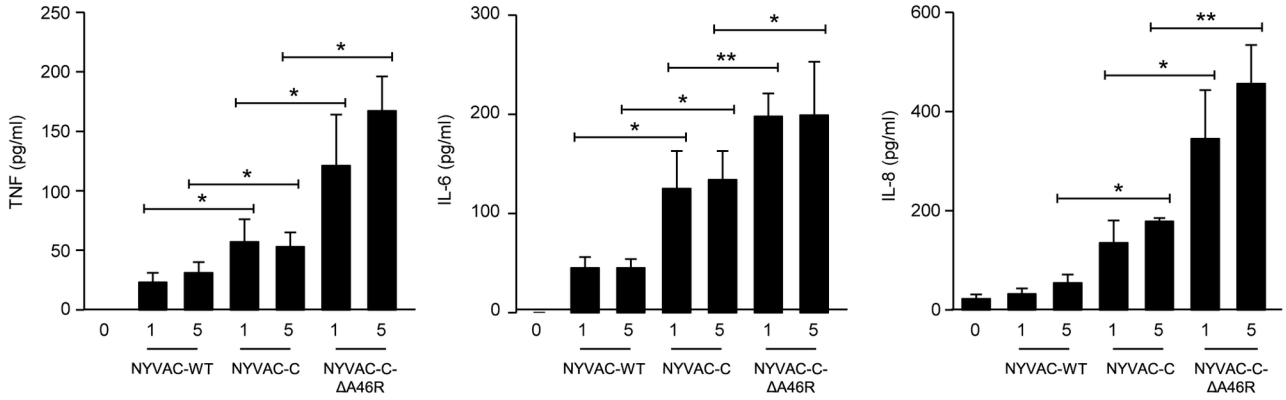

Figure 2. Deletion of *A46R* gene from NYVAC-C enhances innate immune responses. Human macrophages were mock-infected (0) or infected with NYVAC-WT, NYVAC-C or NYVAC-C-Δ46R (1 or 5 PFU/cell). 24 hours later, cell-free supernatants were collected to quantify the concentrations of TNF and IL-6 by bioassay and of IL-8 by ELISA. Data are means ± SD of duplicates and are representative of three independent experiments. * $p<0.05$, ** $p<0.005$.

doi: 10.1371/journal.pone.0074831.g002

NYVAC-C. Since the contribution of DNA priming is the same for NYVAC-C and NYVAC-C-ΔA46R immunization groups, the differences observed should be attributed to the *A46R* deletion.

Deletion of the viral gene *A46R* impacts on the HIV-1-specific CD8 T cell memory phase of the immune response

Phenotypic analysis of memory vaccine-induced T cell immune responses was performed by polychromatic ICS assay 53 days after the last immunization. Splenocytes from immunized mice were stimulated *ex vivo* for 6 hours with the HIV-1 peptide pools Env, Gag and GPN and stained with specific antibodies to identify T cell lineage (CD3, CD4 and CD8), degranulation (CD107a), responding cells (IL-2, IFN-γ and TNF-α) as well as memory stages (CD127 and CD62L).

The magnitudes of the memory HIV-1-specific CD4 or CD8 T cell responses, determined as the sum of the individual responses obtained for Env, Gag and GPN peptide pools, were significantly higher in the groups boosted with the parental NYVAC-C or with the NYVAC-C-ΔA46R deletion mutant than in the control group immunized with NYVAC-WT ($p<0.001$) (Figure 4A).

The magnitude of the HIV-1-specific CD4 T cell response in the group immunized with DNA-C/NYVAC-C-ΔA46R was similar to that obtained in the group DNA-C/NYVAC-C and in both cases it was mainly directed against Env. On the other hand, the $CD8^+$ T cell responses were higher in magnitude and *A46R* gene deletion clearly induced a significant enhancement in the magnitude of the $CD8^+$ T cell responses against Env and GPN ($p<0.001$). Representative functional profiles of Env-induced CD8 T cell responses are shown in Figure S1.

HIV-specific CD8 T cell responses were polyfunctional in both immunization groups with 75% of CD8 T cells exhibiting two, three or four functions. CD8 T cells producing CD107a+ IFN-γ+TNF-α, CD107a+ IFN-γ+IL-2+TNF-α, CD107a+ TNF-α or only CD107a were the most representative populations induced (Figure 4B).

Since previous studies have shown that CD127 and CD62L define functionally distinct populations of memory antigen-specific T cells [51], we characterized the differentiation stages of the responding CD8 T cells into central memory (TCM; $CD127^+CD62L^+$), effector memory (TEM; $CD127^+CD62L^-$) or effector (TE; $CD127^-CD62L^-$) populations. As shown in Figure 4C, about 60% of the HIV-specific CD8 T cells were of TEM phenotype in the DNA-C/NYVAC-C and DNA-C/NYVAC-C-ΔA46R groups.

Overall, these results indicate that deletion of *A46R* gene from NYVAC-C genome improved the magnitude of the HIV-1-specific memory CD8 T cell immune response and maintained the polyfunctional profile and memory differentiation pattern observed with the parental NYVAC-C.

Figure 3

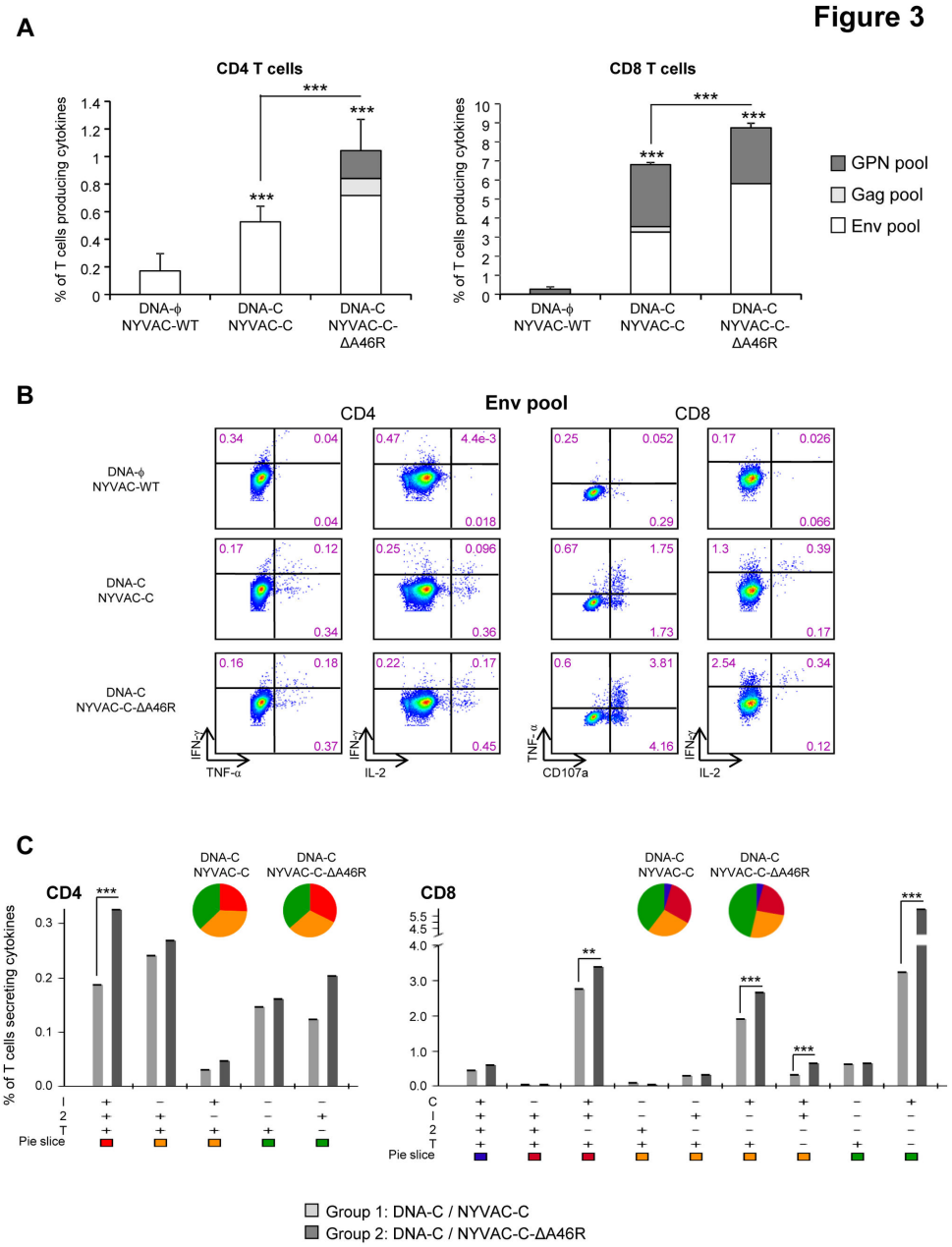

Figure 3. Adaptive HIV-specific T cell immune responses elicited by *A46R* deletion mutant in the spleen of BALB/c mice in heterologous prime/boost immunization protocol. (A) Magnitude of the vaccine-specific CD4 or CD8 T cell response. The HIV-specific CD4 or CD8 T cells were measured 10 days after the last immunization by ICS assay following stimulation of splenocytes derived from immunized animals (n=4) with the different HIV peptide pools. The total value in each group represents the sum of the percentages of $CD4^+$ or $CD8^+$ T cells secreting IFN-γ and/or IL-2 and/or TNF-α (CD4) or CD107a and/or IFN-γ and/or IL-2 and/or TNF-α (CD8) against all HIV peptide pools. All data are background-subtracted. *** $p<0.001$. *p* value indicates significantly higher responses compared to parental group or between DNA-C/NYVAC-C-ΔA46R and DNA-C/NYVAC-C immunization groups. (B) Flow cytometry profiles of vaccine-induced CD4 or CD8 T cell responses against Env pool. (C) Functional profile of the adaptive HIV-specific CD4 or CD8 T cell response in the different immunization groups. The possible combinations of the responses are shown on the *x* axis, whereas the percentages of the functionally distinct cell populations within the total CD4 or CD8 T cell population are shown on the *y* axis. Combinations that did not contribute significantly to the functional profile are not shown. Responses are grouped and colour-coded on the basis of the number of functions. The non-specific responses obtained in the control group DNA-ϕ/NYVAC-WT were subtracted in all populations. ** p<0.005, *** $p<0.001$. *p* values indicate significantly higher responses compared to DNA-C/NYVAC-C immunization group.

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Figure 4

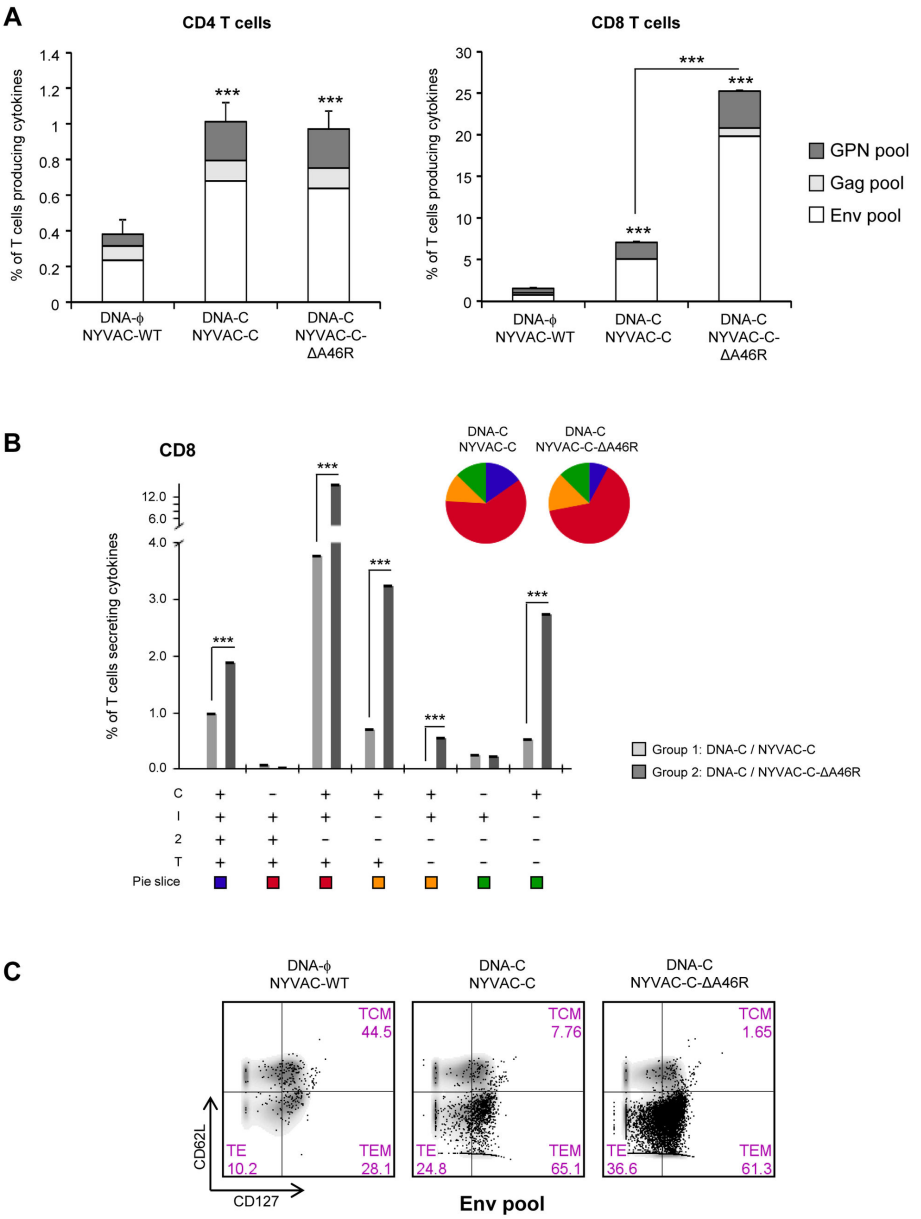

Figure 4. Memory HIV-specific T cell immune responses elicited by *A46R* deletion mutant in the spleen of BALB/c mice after prime/boost immunization. (A) Magnitude of the vaccine-specific CD4 or CD8 T cell responses. The HIV-specific CD4 or CD8 T cells were measured 53 days after the last immunization by ICS assay following stimulation of splenocytes derived from immunized animals (n=4) with the different HIV peptide pools. The total value in each group represents the sum of the percentages of $CD4^+$ or $CD8^+$ T cells secreting IFN-γ and/or IL-2 and/or TNF-α (CD4) or CD107a and/or IFN-γ and/or IL-2 and/or TNF-α (CD8) against all HIV peptide pools. All data are background-subtracted. *** $p<0.001$. *p* value indicates significantly higher responses compared to parental group or between DNA-C/NYVAC-C and DNA-C/NYVAC-C-ΔA46R immunization groups. (B) Functional profile of the memory HIV-specific CD8 T cell response in the different immunization groups. The possible combinations of the responses are shown on the *x* axis, whereas the percentages of the functionally distinct cell populations within the total CD8 T cell population are shown on the *y* axis. Combinations that did not contribute significantly to the functional profile are not shown. Responses are grouped and colour-coded on the basis of the number of functions. *** $p<0.001$. *p* values indicate significantly higher responses compared to DNA-C/NYVAC-C immunization group. (C) Phenotypic profile of memory HIV-specific CD8 T cells. Representative FACS plots showing the percentage of Env-specific CD8 T cells with central memory (TCM; $CD127^+CD62L^+$), effector memory (TEM; $CD127^+CD62L^-$) or effector (TE; $CD127^-CD62L^-$) phenotype.

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Figure 5

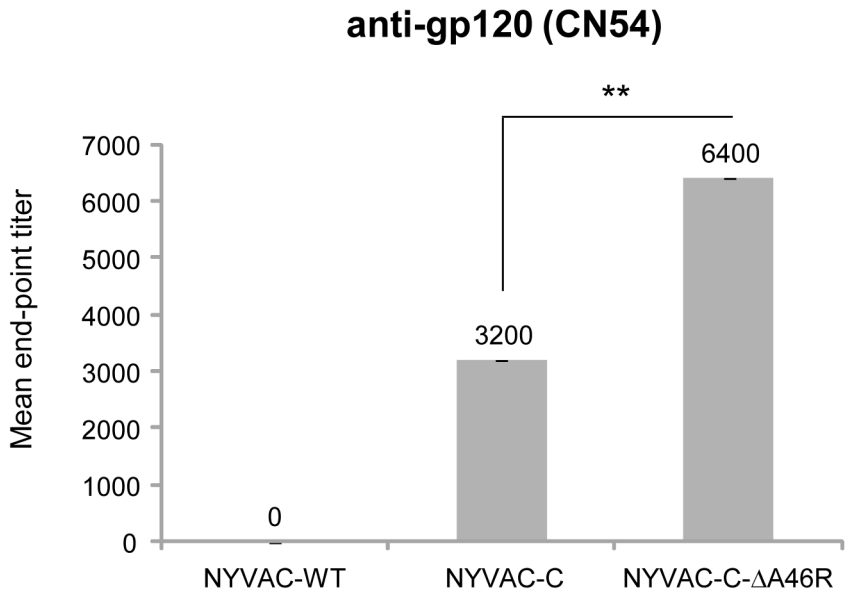

Figure 5. Memory humoral immune response elicited by the *A46R* deletion mutant against HIV-1 gp120 protein. Levels of Env-specific IgG binding antibodies were measured in serum from naïve and immunized mice at day 68. The values represent the mean antibodies titer for each group. ** p<0.005.

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Deletion of the viral gene *A46R* in NYVAC-C enhances the anti-gp120 humoral response during the memory phase

Since cells infected with NYVAC-C release monomeric gp120 [48], we also evaluated the impact of the deletion of viral gene *A46R* on the humoral response at day 68. We quantified by ELISA the Env-specific IgG titers against the purified gp120 protein from the HIV-1 isolate CN54 (clade C). As shown in Figure 5, the IgG titer obtained in the pool of sera of animals immunized with NYVAC-C-ΔA46R is significantly higher (p<0.005) than the titer obtained in the sera of animals immunized with NYVAC-C indicating that deletion of the viral gene *A46R* enhances the humoral response induced in mice during the memory phase.

Deletion of the viral gene *A46R* impacts on the anti-vector CD8 T cell adaptive and memory phases of the immune response

Vaccine-induced anti-vector T cell immune response was measured 10 and 53 days after the last immunization by polychromatic ICS assay. Splenocytes from immunized animals were stimulated *ex vivo* for 6 hours with VACV E3 peptide, which is specific for CD8 T cells [52]. During the adaptive phase of the immune response, the magnitude of the E3-specific CD8 T cell response was significantly lower in the DNA-C/NYVAC-C and DNA-C/NYVAC-C-ΔA46R immunized groups than in the control group immunized with DNA-ϕ/NYVAC-WT (p<0.001) (Figure 6A). No statistical differences were observed between the DNA-C/NYVAC-C and DNA-C/NYVAC-C-ΔA46R groups. E3-specific CD8 T cell responses were polyfunctional in all the immunization groups with almost 50% of $CD8^+$ T cells exhibiting two, three or four functions. CD8 T cells producing only CD107a were the most representative population induced (Figure 6B). During the memory phase, the magnitude of the E3-specific CD8 T cell response in both immunization groups DNA-C/NYVAC-C and DNA-C/NYVAC-C-ΔA46R was significantly lower than that obtained in the control group DNA-ϕ/NYVAC-WT (p<0.001) and the magnitude of the E3-specific CD8 T cell response observed in the group DNA-C/NYVAC-C-ΔA46R was significantly lower than that obtained in the group DNA-C/NYVAC-C (p<0.001) (Figure 7A). E3-specific CD8 T cell responses were polyfunctional in all the immunization groups with almost 90% of $CD8^+$ T cells

exhibiting two, three or four functions. CD8 T cells producing CD107a+ IFN-γ+TNF-α or CD107a+ IFN-γ+IL-2+TNF-α were the most representative populations induced (Figure 7B). Overall, these results indicate that deletion of *A46R* gene from NYVAC-C genome reduced the magnitude of the VACV E3-specific adaptive and memory CD8 T cell immune response but maintained the polyfunctional profile observed with the parental NYVAC-C. Since adaptive and memory immune responses to HIV antigens were enhanced by the *A46R* deletion mutant (Figures 3 and 4), the reduced T cell immune response induced by the E3 peptide indicates an immunodominance of HIV antigens.

Discussion

Development of non-replicating VACV vectors with enhanced immunogenicity against foreign expressed antigens is a major goal in the poxvirus field, aiming at the application of these vectors as HIV/AIDS vaccines. This is in view of the restricted immunogenicity triggered in clinical trials by the parental vectors expressing HIV antigens, like MVA, NYVAC, canarypox and fowlpox [53]. In fact, the reduced efficacy against HIV infection, 31.2%, of the non-replicating canary poxvirus vector combined with gp120 protein in the RV144 clinical trial [1], highlighted the need of novel poxvirus vectors with improved immunogenicity. With regard to non-replicating poxvirus vectors, different strategies have been pursued to enhance their potency, like the combination of heterologous vectors, use of co-stimulatory molecules and disruption of viral genes encoding immunosuppressive molecules [53]. The latter strategy provides the additional advantage that the immunomodulatory role of a viral gene can be easily quantified in an organism.

A number of MVA deletion mutants in viral immune modulators have been generated to date and tested in mice [54,55,56,57,58] and macaques [59,60]. These studies have shown that MVA recombinant viruses with a single deletion of viral genes encoding inhibitors of type 1 IFN signalling pathway (*C6L* [55]), apoptosis (*F1L* [56]), IL-18 binding protein (*C12L* [57]) or the uracyl-DNA glycosylase gene (*UDG* [60]), enhanced the overall immune responses to HIV-1 antigens. The HIV-1-specific CD4 and CD8 T cell immune responses were further increased by MVA vectors with deletions of two (*A41L*/*B16R* [54]; or *C6L*/*K7R*; Garcia-Arriaza, submitted) or four [IL-18 binding protein (MVA008L; *C12L*), Toll/IL-1 receptor homolog (MVA159R; *A46R*), CC-chemokine binding protein (MVA153L; *B7R*) and secreted IL-1β receptor (MVA184R; *B16R*)] immunomodulatory genes [59], while an additional fifth deletion of the uracyl-DNA glycosylase gene (MVA101R) decreased the responses [59]. Similarly, NYVAC vectors with single or double deletions in VACV genes *B19R* and *B8R* encoding type I and type II IFN binding proteins, respectively, increased the immune responses to HIV antigens in the mouse model [61].

In an effort to uncover the role of VACV genes as immune modulators and search for potential applications of these vectors in the development of optimized vaccines, in this investigation we showed that deletion of the viral TLR inhibitor *A46R* gene in the NYVAC-C genome has no effect on the replication capacity of the virus in CEF cells but triggers expression of immunoregulatory genes in infected macrophages. NYVAC-C also enhances, though to a lesser extent, the production of these proinflammatory cytokines and chemokines compared to NYVAC-WT indicating that the expression of HIV-1 antigens has an effect on innate immune cells. The impact on antigen-presenting cells of the expression of HIV antigens from an attenuated poxvirus vector has been previously reported by using microarray technology in human dendritic cells infected with an MVA-based recombinant virus expressing gp120 and GPN from clade B [62].

Significantly, in mice immunized following a DNA prime/ NYVAC boost protocol, the deletion mutant NYVAC-C-ΔA46R enhanced HIV-specific T cell immune responses. Both CD4 and CD8 T cells specific for HIV antigens were activated. In the adaptive phase, the magnitudes of the HIV-1-specific CD4 or CD8 T cell responses in the group immunized with NYVAC-C-ΔA46R were significantly higher than those obtained in the group DNA-C/NYVAC-C (p<0.001), maintaining the polyfunctional profile observed with the parental NYVAC-C. In the memory phase, deletion of *A46R* gene from NYVAC-C genome improved again the magnitude of the HIV-1-specific memory CD8 T cell immune response, while both the polyfunctional profile and memory differentiation pattern observed were similar as those obtained with the parental NYVAC-C. The main phenotype of the memory response was TEM, which is of immunological relevance as this phenotype has been correlated with protection in the macaque-SIV model [63,64].

This enhanced HIV-specific T cell immune response is in contrast with the lack or reduced effect of *A46R* deletion on VACV E3-specific T cell responses during adaptive or memory phases of the immune response, respectively (Figures 6 and 7). The absence or decrease of immune stimulatory effect observed when E3 was used to stimulate mouse splenocytes in comparison with the increased responses against HIV antigens is likely to be related to the immune dominance of the HIV antigens versus the viral E3 peptide and such immunodominance may be due to the effect of the priming with a DNA encoding the HIV-1 antigens and also to the fact that NYVAC-C expresses E3 under its natural early promoter while the HIV antigens are expressed at early and late times from a strong synthetic early/late promoter. Since both explanations can be applied to NYVAC-C or NYVAC-C-ΔA46R-induced immune responses, the lower E3-specific CD8 T cell adaptive and memory immune responses elicited by NYVAC-C-ΔA46R deletion mutant compared with that induced by NYVAC-C are inversely correlated with the higher HIV-1-specific CD8 T cell responses triggered by the *A46R* deletion mutant. A similar trend for E3 response in relation to foreign expressed antigens has been observed for other recombinant VACV vectors [65]. Therefore, a reduction of immune responses to NYVAC-C-ΔA46R vector antigens has the additional vaccine advantage that HIV antigens are favoured over viral antigens, thus enhancing the specific immune responses to HIV.

Since humoral response against HIV antigens has been described to be important for protection against HIV acquisition

Figure 6

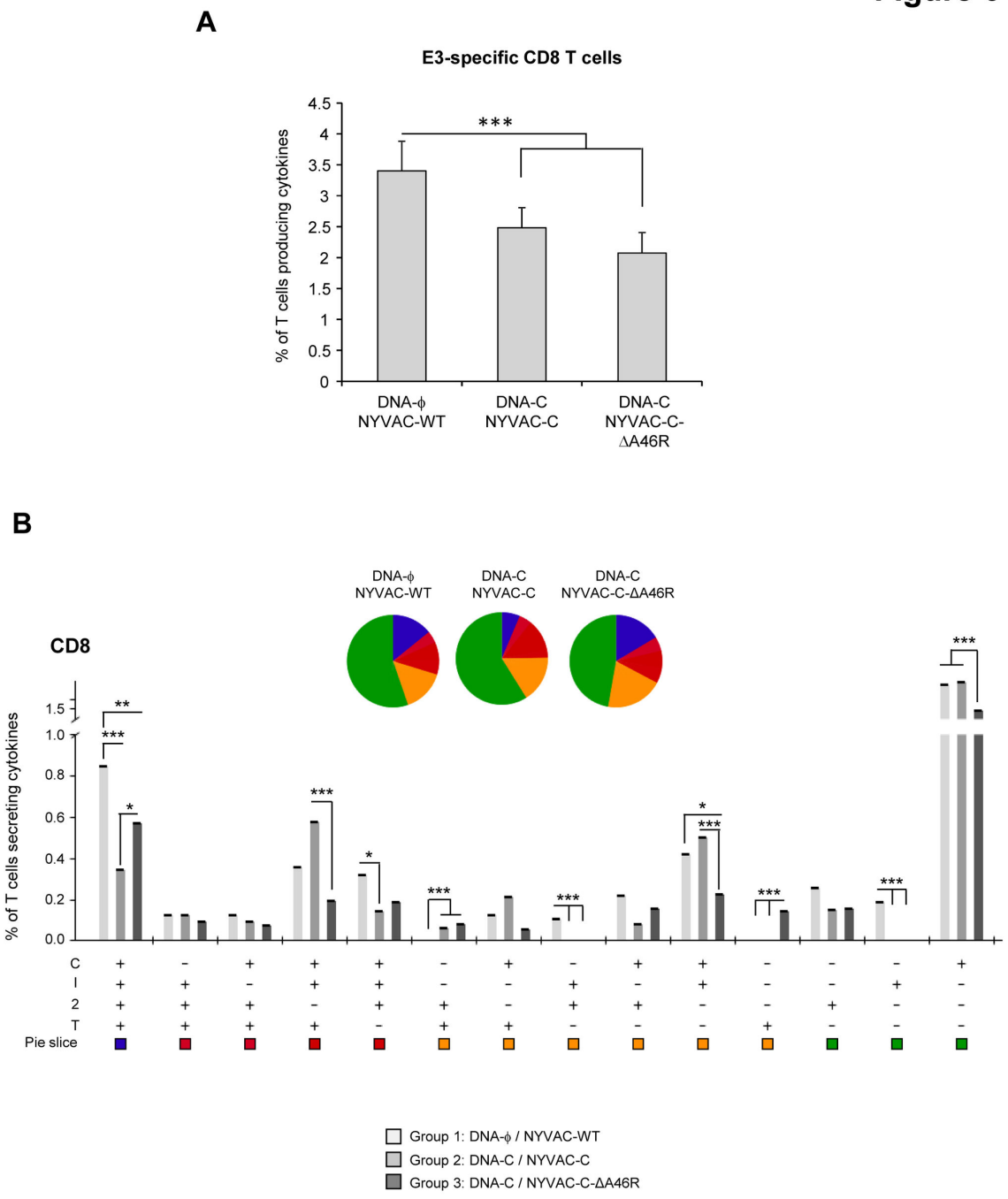

Figure 6. Adaptive VACV vector-specific T cell immune responses elicited by *A46R* deletion mutant in the spleen of BALB/c mice after prime/boost immunization. (A) Magnitude of the VACV-specific CD8 T cell response. The VACV-specific CD8 T cells were measured 10 days after the last immunization by ICS assay following stimulation of splenocytes derived from immunized animals (n=4) with VACV E3 peptide. The total value in each group represents the sum of the percentages of $CD8^+$ T cells secreting CD107a and/or IFN-γ and/or IL-2 and/or TNF-α against E3 peptide. All data are background-subtracted. *** $p<0.001$. *p* value indicates significantly higher response compared to DNA-C/NYVAC-C and DNA-C/NYVAC-C-ΔA46R immunization groups. (B) Functional profile of the VACV-specific CD8 T cell response in the different immunization groups. The possible combinations of the responses are shown on the *x* axis, whereas the percentages of the functionally distinct cell populations within the total CD8 T cell population are shown on the *y* axis. Combinations that did not contribute significantly to the functional profile are not shown. Responses are grouped and colour-coded on the basis of the number of functions. * p<0.05, ** p<0.005, *** $p<0.001$.

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Figure 7

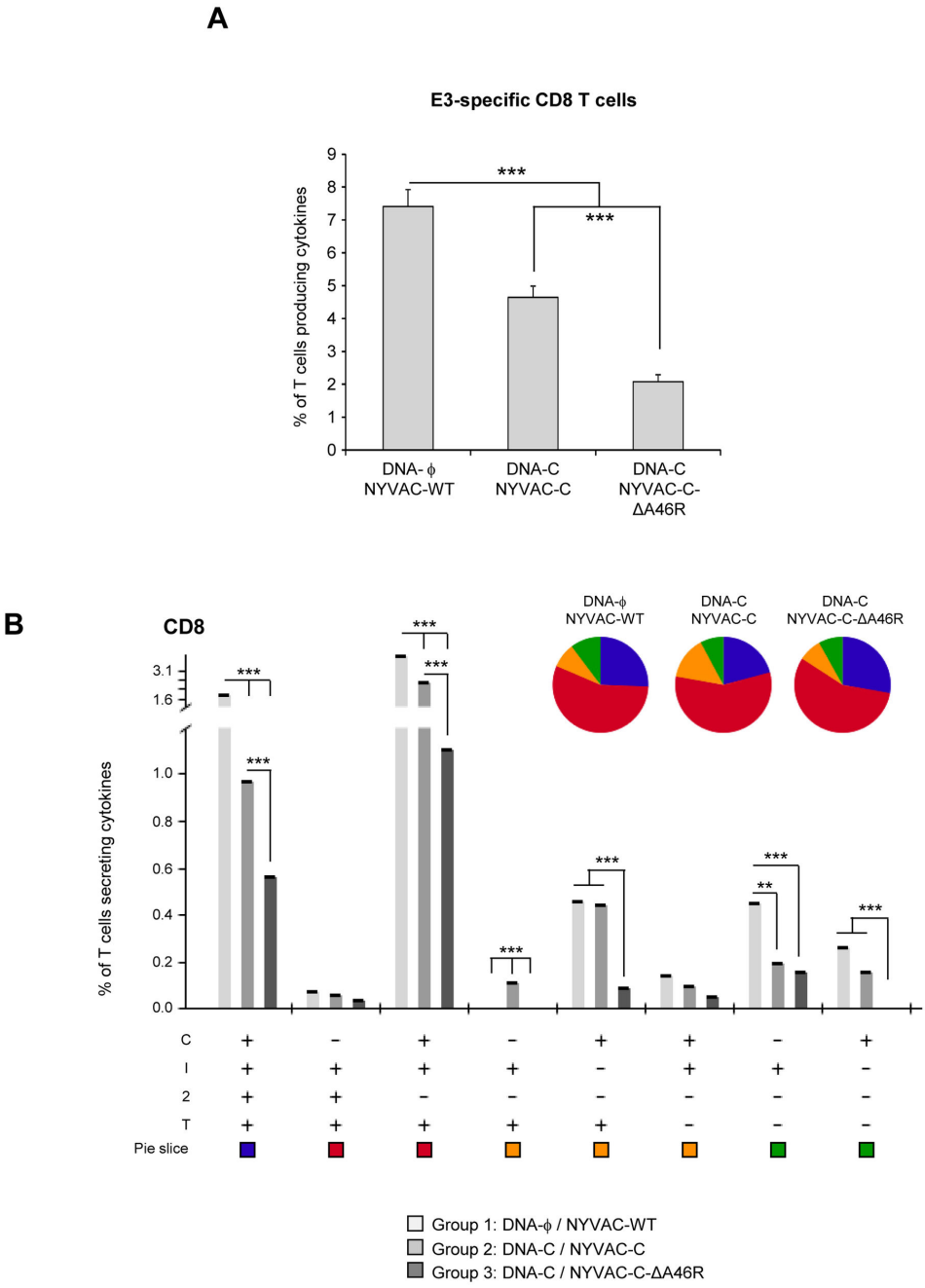

Figure 7. Memory VACV vector-specific T cell immune responses elicited by *A46R* deletion mutant in the spleen of BALB/c mice after prime/boost immunization. (A) Magnitude of the VACV-specific CD8 T cell response. The VACV-specific CD8 T cells were measured 53 days after the last immunization by ICS assay following stimulation of splenocytes derived from immunized animals (n=4) with VACV E3 peptide. The total value in each group represents the sum of the percentages of $CD8^+$ T cells secreting CD107a and/or IFN-γ and/or IL-2 and/or TNF-α against E3 peptide. All data are background-subtracted. *** $p<0.001$. *p* values indicate significantly higher response compared to DNA-C/NYVAC-C and DNA-C/NYVAC-C-ΔA46R immunization groups or between DNA-C/NYVAC-C and DNA-C/NYVAC-C-ΔA46R groups. (B) Functional profile of the VACV-specific CD8 T cell response in the different immunization groups. The possible combinations of the responses are shown on the *x* axis, whereas the percentages of the functionally distinct cell populations within the total CD8 T cell population are shown on the *y* axis. Combinations that did not contribute significantly to the functional profile are not shown. Responses are grouped and colour-coded on the basis of the number of functions. ** $p<0.005$, *** $p<0.001$.

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[1], we also evaluated the presence of anti-gp120 antibodies in the serum of immunized animals. This analysis showed an enhanced anti-gp120 humoral response in the mice immunized with NYVAC-C-ΔA46R deletion mutant suggesting that the deletion of *A46R* gene is also able to modulate positively the humoral response against gp120.

How deletion of *A46R* impacts on the immune response of NYVAC-C? As previously described, A46 impairs TLR signalling by targeting the TIR domain of the adaptors MyD88, Mal, TRIF and TRAM disrupting Receptor: Adaptor TIR interactions [37]. Hence, deleting *A46R* in NYVAC restores TLR signalling upon viral infection, enhancing the expression of proinflammatory molecules, which in turn will enhance T cell activation. According to the intraperitoneal route used in the present study, the effect of *A46R* gene deletion on immunogenicity against HIV-1 antigens should be explained by the effect of TLR signalling restoration in the cell types present in the peritoneal cavity (mainly B cells, macrophages and granulocytes and, to a lesser extent, T cells [66]). In this context, the increased secretion of proinflammatory cytokines and chemokines by NYVAC-C-ΔA46R-infected macrophages could induce an enhanced recruitment of immature DCs and lymphocytes, generating an appropriate environment for the uptake and presentation of HIV-1 antigens to T cells. Immature NYVAC-C-ΔA46R-infected DCs can also migrate to the lymph nodes, maturing in route, and activate HIV-1-specific T cells enhancing the overall immunogenicity against HIV antigens. According to this, it has been previously reported that the total number of cells in the lungs of mice immunized intranasally with a VACV *A46R* deletion mutant was increased on day 2 post-infection compared with parental virus whereas on days 5 and 8 was reduced [37]. Since the main innate sensors of VACV vectors are TLR2 [15,16,17], TLR2-TLR6-MyD88, MDA-5/IPS-1 and NALP3 inflammasome [47] and *A46R* targets the TIR domain of the adaptors MyD88, Mal, TRIF and TRAM [37], our findings of enhanced production of TNF, IL-6 and IL-8 in conjunction with an increase in the magnitude of CD4 and CD8 T cell immune responses to HIV antigens and an enhanced gp120-specific humoral response, reveal that *A46R* plays an important role as immune modulator. This observation, in combination with the biochemical data on the mode of action of A46, establishes the immunological role of VACV *A46R* on T and B cell responses.

Materials and Methods

Ethics statement

The animal studies were approved by the Ethical Committee of Animal Experimentation (CEEA-CNB) of Centro Nacional de Biotecnologia (CNB-CSIC, Madrid, Spain) in accordance with national and international guidelines and with the Royal Decree (RD 1201/2005) (Permit numbers: 152/07 and 080030).

Cells and viruses

African green monkey kidney cells (BSC-40; American Type Culture Collection, Manassas, VA) and primary chicken embryo fibroblast cells (CEF; Intervet, s.a, Salamanca, Spain) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 10% newborn calf serum (NCS) for BSC-40 cells or 10% fetal calf serum (FCS) for CEF cells. The human monocytic THP-1 cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium containing 2 mM L-glutamine, 50 µM 2-mercaptoethanol, 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 10% FCS. Cells were maintained in a humidified air 5% CO_2 atmosphere at 37°C. The poxvirus strains used in this work included the genetically attenuated vaccinia virus-based vector NYVAC-WT and the recombinant NYVAC-C expressing gp120 as a cell-released product and Gag-Pol-Nef as an intracellular polyprotein from the clade C CN54 HIV-1 isolate [48], used as the parental vector for the generation of the *A46R* deletion mutant. Virus infections were performed with 2% NCS or FCS. All viruses were grown in primary CEF cells, similarly purified through two 36% (w/v) sucrose cushions and the virus titers were determined by immunostaining plaque assay in BSC-40 cells as previously described [67]. The titer determinations of the different viruses were performed at least three times.

Construction of plasmid transfer vector pGem-RG-A46R wm

The plasmid transfer vector pGem-RG-A46R wm, used for the construction of the recombinant virus NYVAC-C-ΔA46R, with *A46R* ORF deleted, was obtained by the sequential cloning of *A46R* recombination flanking sequences into the plasmid pGem-Red-GFP wm, containing dsRed2 and rsGFP genes as fluorescent markers, and previously described [68]. NYVAC genome was used as the template to amplify the left flank of *A46R* gene (432 bp) with oligonucleotides LFA46R-Aat (5´-CACGATGACGTCAGAGGAGTTAT-3´) (AatII site underlined) and LFA46R-Xba (5´-CGTATGTCTAGATTATTTTGCTGAG-3´) (XbaI site underlined). This left flank was digested with AatII and XbaI and cloned into plasmid pGem-Red-GFP wm previously digested with the same restriction enzymes to generate pGem-RG-LFsA46R wm (4939 bp). The repeated left flank of *A46R* gene (432 bp) was amplified by PCR from NYVAC genome with oligonucleotides LFA46R'-Eco (5´-CACGATGAATTCAGAGGAGTTAT-3´) (EcoRI site underlined) and LFA46R'-Cla (5´-CGTATGATCGATT TATTTTGCTGAG-3´) (Cla I site underlined), digested with EcoRI and Cla I and inserted into the EcoRI / Cla I-digested pGem-RG-LFsA46R wm to generate pGem-RG-LFdA46R wm (5330 bp). The right flank of *A46R* gene (360 bp) was amplified by PCR from NYVAC genome with oligonucleotides RFA46R-Cla (5´-CTGAGAATCGATAGGATGAATTTG-3´) (Cla I site underlined) and RFA46R-Bam (5´-ATTTAAGGATCCAGAACGGCAAC-3´) (BamHI site underlined), digested with Cla I and BamHI and inserted into the Cla I / BamHI-digested pGem-RG-LFdA46R wm. The resulting plasmid pGem-RG-A46R wm (5660 bp; Figure S2) was confirmed by DNA sequence analysis and directs the deletion of *A46R* gene from NYVAC-C genome.

Construction of NYVAC-C-ΔA46R deletion mutant

The deletion mutant NYVAC-C-ΔA46R was constructed using dsRed2 and rsGFP as fluorescent markers. 3 x 10^6

BSC-40 cells were infected with 0.01 PFU/cell of NYVAC-C and transfected 1 hour later with 6 µg DNA of plasmid pGem-RG-A46R wm using Lipofectamine (Invitrogen) according to the manufacturer's recommendations. Forty-eight hours post-infection, the cells were harvested, lysed by freeze-thaw cycling, sonicated and used for recombinant virus screening. Deletion mutant was selected from progeny virus by consecutive rounds of plaque purification in BSC-40 cells during which plaques were screened for Red2/GFP fluorescence. In the first three passages, viruses from selected plaques expressed both fluorescent proteins, while in the next two passages viral progeny from selected plaques expressed only one fluorescent marker (Red2). In the last two passages (seven passages in total), viruses from selected plaques do not express any marker due to the loss of the fluorescent marker by homologous recombination within the repeated flanking DNA sequences. The resulting NYVAC-C-ΔA46R virus was expanded in BSC-40 cells and the crude preparation obtained was used for the propagation of the virus in large cultures of primary chicken fibroblasts (CEF) followed by virus purification through two 36% (w/v) sucrose cushions and titrated by immunoplaque assay in BSC-40 cells.

PCR analysis of NYVAC-C-ΔA46R deletion mutant

To test the identity and purity of the recombinant virus NYVAC-C-ΔA46R, viral DNA was extracted from BSC-40 cells infected at 5 PFU/cell with NYVAC-WT or NYVAC-C-ΔA46R. Cell membranes were disrupted using sodium dodecyl sulphate (SDS) followed by proteinase K treatment (0.2 mg/ml proteinase K in 50 mM Tris-HCl pH 8, 100 mM EDTA pH 8, 100 mM NaCl and 1% SDS for 1 hour at 55°C) and phenol extraction of viral DNA. Primers LFA46R-Aat and RFA46R-Bam spanning *A46R* flanking regions were used for PCR analysis of *A46R* locus. The amplification reactions were carried out with Platinum Taq DNA polymerase (Invitrogen) according to the manufacturer's recommendations. The correct sequence of deleted *A46R* locus was confirmed by DNA sequence analysis.

Expression of HIV-1 proteins gp120 and GPN

To test the correct expression of HIV-1 antigens by the *A46R* deletion mutant, monolayers of BSC-40 cells were mock-infected or infected at 5 PFU/cell with NYVAC-WT, NYVAC-C or NYVAC-C-ΔA46R. At 24 hours post-infection, cells were lysed in Laemmli buffer, cells extracts fractionated by 8% SDS-PAGE and analyzed by Western-blot using the polyclonal anti-gp120 antibody (Centro Nacional de Biotecnología; diluted 1:3000) or the polyclonal anti-gag p24 serum (ARP 432, NIBSC, Centralised Facility for AIDS reagent, UK; diluted 1:1000) to evaluate the expression of gp120 and GPN proteins, respectively. The anti-rabbit-HRPO (SIGMA; diluted 1:5000) was used as secondary antibody. The immunocomplexes were detected by enhanced chemiluminescence (ECL, GE Healthcare).

Analysis of virus growth

To determine virus growth profiles, monolayers of CEF cells grown in 12-well plates were infected in duplicate at 0.01 PFU/cell with NYCAC-C or NYVAC-C-ΔA46R deletion mutant. Following virus adsorption for 60 min at 37°C, the inoculum was removed. The infected cells were washed once with DMEM without serum and incubated with fresh DMEM containing 2% FCS at 37°C in a 5% CO_2 atmosphere. At different times post-infection (0, 24, 48 and 72 hours), cells were harvested by scraping (lysates at 5 x 10^5 cells/ml), freeze-thawed three times and briefly sonicated. Virus titers in cell lysates were determined by immunostaining plaque assay in BSC-40 cells using rabbit polyclonal anti-vaccinia virus strain WR (Centro Nacional de Biotecnología; diluted 1:1000), followed by anti-rabbit-HRPO (SIGMA; diluted 1:1000).

Measurement of cytokine production by macrophages

THP-1 cells were differentiated into macrophages by treatment with 0.5 mM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 24 h. The medium was changed and cells were either mock-infected or infected with 1 or 5 PFU/cell of NYVAC-WT, NYVAC-C or NYVAC-C-Δ46R. Cell-free supernatants were collected after 6 hours to quantify the concentrations of TNF, IL-6 and IL-8. The concentrations of human IL-8 (BD Biosciences) in cell-culture supernatants were measured by ELISA as previously described [47]. TNF and IL-6 concentrations were measured by bioassay as described elsewhere [69].

DNA vectors

The two DNA constructs expressing the HIV-1 $_{CN54}$gp120 (pcDNA- $_{CN54}$gp120) and HIV-1 $_{CN54}$Gag-Pol-Nef (GPN) polyprotein (pcDNA- $_{CN54}$GPN) have been previously reported [48]. Plasmids were purified using Maxi-prep purification kits (Qiagen) and diluted for injection in endotoxin-free PBS.

Peptides

The HIV-1 peptide pools Gag-1, Gag-2, Env-1, Env-2, GPN-1, GPN-2, GPN-3 and NEF were provided by the EuroVacc Foundation and were previously described [48]. They spanned the HIV-1 Env, Gag, Pol and Nef antigens from clade C included in the immunogens as consecutive 15-mers overlapping by 11 amino acids. For immunological analyses we grouped the pools as follows: Env pool (Env-1+Env-2), Gag pool (Gag-1+Gag-2) and GPN pool (GPN-1+GPN-2+GPN-3+NEF). The VACV $E3_{140-148}$ peptide (VGPSNSPTF; CNB), previously described as immunodominant epitope in BALB/c mice [52], was used to detect the anti-vector cellular immune response.

Mouse immunization schedule

BALB/c mice (6-8 weeks old) were purchased from Harlan. For the heterologous DNA prime/NYVAC boost immunization protocol performed to assay the immunogenicity of NYVAC-C-ΔA46R deletion mutant, groups of animals (n=8) received 100 µg of DNA-C (50 µg of pcDNA- $_{CN54}$gp120 + 50 µg of pcDNA- $_{CN54}$GPN) or 100 µg of DNA-ϕ (100 µg of pcDNA) by intramuscular route (i.m.). Two weeks later, animals were immunized with 1 x 10^7 PFU of NYVAC-WT, NYVAC-C or NYVAC-C-ΔA46R by intraperitoneal route (i.p.). Mice

immunized with sham DNA (DNA-ϕ) followed by NYVAC-WT boost were used as control group. At 10 and 53 days after the last immunization, 4 mice in each group were sacrificed and spleens processed for Intracellular Cytokine Staining (ICS) assay to measure the adaptive and memory cellular immune responses against HIV-1 antigens, respectively. Two independent experiments have been performed for the different groups.

Intracellular Cytokine Staining assay (ICS)

The magnitude, polyfunctionality and phenotype of the HIV-specific T cell responses were analyzed by ICS. After an overnight rest, 4 x 10^6 splenocytes (depleted of red blood cells) were seeded on 96-well plates and stimulated during 6 hours in complete RPMI 1640 media supplemented with 10% FCS containing 1 µl/ml GolgiPlug (BD Biosciences), anti-CD107a-Alexa 488 (BD Biosciences) and 5 µg/ml of the different HIV peptide pools. At the end of the stimulation period, cells were washed, stained for the surface markers, fixed and permeabilized (Cytofix/Cytoperm Kit; BD Biosciences) and stained intracellularly using the appropriate fluorochromes. Dead cells were excluded using the violet LIVE/DEAD stain kit (Invitrogen). For functional analyses the following fluorochrome-conjugated antibodies were used: CD3-PE-CF594, CD4-APC-Cy7, CD8-V500, IFN-γ-PE-Cy7, IL-2-APC and TNF-α-PE (all from BD Biosciences). In addition, for phenotypic analyses the following antibodies were used: CD62L-Alexa 700 (BD Biosciences) and CD127-PerCP-Cy5.5 (eBioscience). Cells were acquired using a GALLIOS flow cytometer (Beckman Coulter). Analyses of the data were performed using the FlowJo software version 8.5.3 (Tree Star, Ashland, OR). The number of lymphocyte-gated events ranged between 1 x 10^5 and 1 x 10^6. After gating, Boolean combinations of single functional gates were then created using FlowJo software to determine the frequency of each response based on all possible combinations of cytokine expression or all possible combinations of differentiation marker expression. For each population, background responses detected in the non-stimulated control samples were subtracted from those detected in stimulated samples for every specific functional combination and the percentages of cells producing cytokines obtained in the DNA-ϕ/NYVAC-WT control populations were also subtracted in all the groups in order to remove the non-specific responses detected as background. Only positive responses are represented.

Antibody measurement by ELISA

Binding antibodies to Env protein in serum were determined by enzyme-linked immunosorbent assay (ELISA) as previously described [48]. Serum samples from naïve and immunized mice were serially 2-fold diluted in duplicate and reacted against 2 µg/ml of the recombinant CN54gp120 purified protein (ARP683, HIV-1 CN54gp120 clade C; EU Programme EVA from the Centre for AIDS Reagents). The antibody titer of Env-specific IgG was defined as the last dilution of serum that resulted in 3 times the mean optical density at 450 nm of the naïve control.

Data analysis and statistics

For the statistical analysis of ICS data, we used a novel approach that corrects measurements for the medium response (RPMI) and allows the calculation of confidence intervals and *p* values of hypothesis tests [54,70]. Only antigen responses values significantly higher than the corresponding RPMI are represented and the background for the different cytokines in the unstimulated controls never exceeded 0.05%. Analysis and presentation of distributions was performed using SPICE version 5.1, downloaded from http://exon.niaid.nih.gov [71]. Comparison of distributions was performed using a Student's T test and a partial permutation test as described [71]. All values used for analyzing proportionate representation of responses are background-subtracted. For the statistical analysis of ELISA data, a 1-way ANOVA with Tukey's honestly significant difference criterion as post-hoc analysis was performed.

Supporting Information

Figure S1. Profile of memory HIV-specific T cell immune responses elicited by *A46R* deletion mutant in the spleen of BALB/c mice after prime/boost immunization. Flow cytometry profiles of vaccine-induced CD8 T cell responses against Env pool in splenocytes from immunized animals.
(TIF)

Figure S2. Scheme of construction of the plasmid transfer vector pGem-RG-A46R wm. The plasmid transfer vector pGem-RG-A46R wm was obtained by the sequential cloning of *A46R* recombination flanking sequences into the plasmid pGem-Red-GFP wm, containing dsRed2 and rsGFP genes as fluorescent markers. NYVAC genome was used as the template to amplify the left flank of *A46R* gene by PCR. This left flank was digested with AatII and XbaI and cloned into plasmid pGem-Red-GFP wm previously digested with the same restriction enzymes to generate pGem-RG-LFsA46R wm (4939 bp). The repeated left flank of *A46R* gene was amplified by PCR from NYVAC genome, digested with EcoRI and Cla I and inserted into the EcoRI / Cla I-digested pGem-RG-LFsA46R wm to generate pGem-RG-LFdA46R wm (5330 bp). The right flank of *A46R* gene was amplified by PCR from NYVAC genome, digested with Cla I and BamHI and inserted into the Cla I / BamHI-digested pGem-RG-LFdA46R wm. The resulting plasmid pGem-RG-A46R wm (5660 bp) directs the deletion of *A46R* gene from NYVAC-C genome.
(TIF)

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Author Contributions

Conceived and designed the experiments: BP CEG JD ME. Performed the experiments: BP CEG MD JD. Analyzed the data: COS. Contributed reagents/materials/analysis tools: TR TC GP. Wrote the manuscript: BP CEG TR ME.

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DISCUSSION

4. Discussion

Here we define distinct poxvirus strategies to improve T cell responses to heterologous antigens and we characterize the immune responses elicited by the new attenuated recombinant NYVAC and MVA virus vectors. We identify the immune properties of these vectors, demonstrate a new mechanism for poxvirus-induced immunity, and provide a basis for the design of novel virus vaccine vectors.

4.1 Neutrophil migration

In the first study, we focused on neutrophil recruitment after NFκB activation induced by NYVAC-C ΔA52RΔB15RΔK7R (NYVAC-C-Δ3) infection. Recent studies showed that the neutrophil migration after intradermal and intranasal MVA infection is CCR1-dependent (Duffy et al., 2012; Price et al., 2014). We nonetheless found that after NYVAC-C-Δ3 infection, neutrophil recruitment to the peritoneal cavity is only partially mediated by CCR1 and not by CCR5, the classical receptors of the chemokine macrophage inflammatory protein (MIP)-1 (Kaufmann et al., 2001). We detected higher levels of monocyte chemoattractant protein-1 (MCP-1/CCL2) in peritoneal exudates and sera from NYVAC-C-Δ3-infected mice compared to those from NYVAC-C infected mice. MCP-1/CCL2 is the primary chemokine involved in monocyte recruitment in murine peritonitis (Takahashi et al., 2009), but also participates in neutrophil recruitment (Johnston et al., 1999). In our model, neutrophil recruitment might also be partially mediated by CCR2 (MCP-1/CCL2 receptor), and the combined deletion of CCR1 and CCR2 in NYVAC-C-Δ3-infected mice could lead to peritoneal neutrophil levels similar to those of NYVAC-C-infected mice.

4.2 Neutrophils with antigen-presenting cell (APC) features

When compared to NYVAC-C-infected mice, the peritoneal cavity of NYVAC-C-Δ3-infected mice showed a significant increase in two neutrophil populations (Nα and Nβ). Although the Nβ subset is more susceptible to VACV infection than Nα, we found that the presence of Nβ neutrophils in peritoneal exudates was dependent on the post-infection cytokine/chemokine environment and was not the result of direct neutrophil infection. *In vitro* and *in vivo*, the cytokine/chemokine environment (*e.g.*, presence of GM-CSF; granulocyte-macrophage colony-stimulating factor) polarizes neutrophils to

acquire an APC phenotype (upregulation of MHC class II and the costimulatory molecules CD80/CD86) (Fanger et al., 1997; Oehler et al., 1998), to trigger CD8 T cell activation (Matsushima et al., 2013). In tumor tissue, the cytokine/chemokine milieu (the presence of TGF-β; transforming growth factor β) polarizes tumor-associated neutrophils (TAN) from the anti-tumor N1 to the pro-tumor N2 phenotype, which is characterized by impaired ability to activate CD8 T cells (Fridlender et al., 2009). In tumors treated with SM16 (a TGF-β receptor kinase inhibitor), CD8 T cells showed strong anti-tumor cytolytic effects (Suzuki et al., 2007), and infiltrated neutrophils were more lobulated and hypersegmented than in untreated tumor-bearing controls (Fridlender et al., 2009). In our study, Nβ neutrophils were more lobulated and hypersegmented than the Nα subtype, which confirms neutrophil ability to acquire a distinct phenotype in specific cytokine/chemokine conditions. Although we detected no difference in TGF-β or GM-CSF secretion between these two groups (not shown), APC markers (CD80 and CD86) were upregulated in Nβ cells, which showed a greater capacity to induce antigen-specific CD8 T cell activation than Nα cells.

4.3 Neutrophil-dependent T cell responses

We demonstrated that the presence of Nβ is insufficient to activate naïve CD8 T cells *ex vivo*; in similar conditions, naïve CD8 T cells did not proliferate (not shown). Our data demonstrated that Nβ neutrophils present antigen to effector CD8 T cells to induce their activation and do not prime naïve CD8 T cells to induce proliferation. We speculate that other APC-activating signals such as CD40, which is involved in dendritic cell stimulation of CD8 T cells (Hernandez et al., 2007), could help neutrophil induction of naïve CD8 T cell proliferation. Recent *in vivo* studies demonstrated that neutrophils do not prime vaccinia virus-specific CD8 T cell responses in the absence of myeloid DC (dendritic cells) (Duffy et al., 2012).

Although we showed that neutrophils are involved in the increase in antigen-specific CD8 T cell responses after NYVAC-C-Δ3 infection, this was not the case for the CD4 T cell response. We speculate that neutrophils prime CD4 T cells with difficulty, and probably negatively control CD4 T cell activation, which would explain the reduction in antigen-specific CD4 T cell responses induced in NYVAC-C Δ3-infected mice. Another study indicated that after protein-adjuvant immunization, neutrophils establish very brief contacts with DC in the draining lymph nodes, with a subsequent poor antigen-specific CD4 response; in neutrophil-depleted mice, these authors showed that

the number and periodicity of DC-T cell interactions increased with enhancement of the antigen-specific CD4 response (Yang et al., 2010a), which supports our hypothesis.

An *in vivo* study of the mechanisms of neutrophil-dependent control of CD4 and CD8 T cell responses to vaccinia virus-delivered antigens would be of great interest. In mice, we did not detect GFP-infected neutrophils in secondary lymphoid organs. We speculate that antigen transport results, not from direct neutrophil infection, but rather from neutrophil uptake of apoptotic infected cells in which antigen has been processed (Figure 9). These neutrophils could transport processed antigens and cross-prime naïve CD8 T cells; peritoneal neutrophils efficiently cross-prime naïve CD8 T cells *in vivo* (Beauvillain et al., 2007) and the cross-priming of viral antigens is a robust process able to generate vigorous CD8 T cell responses (Chen et al., 2004).

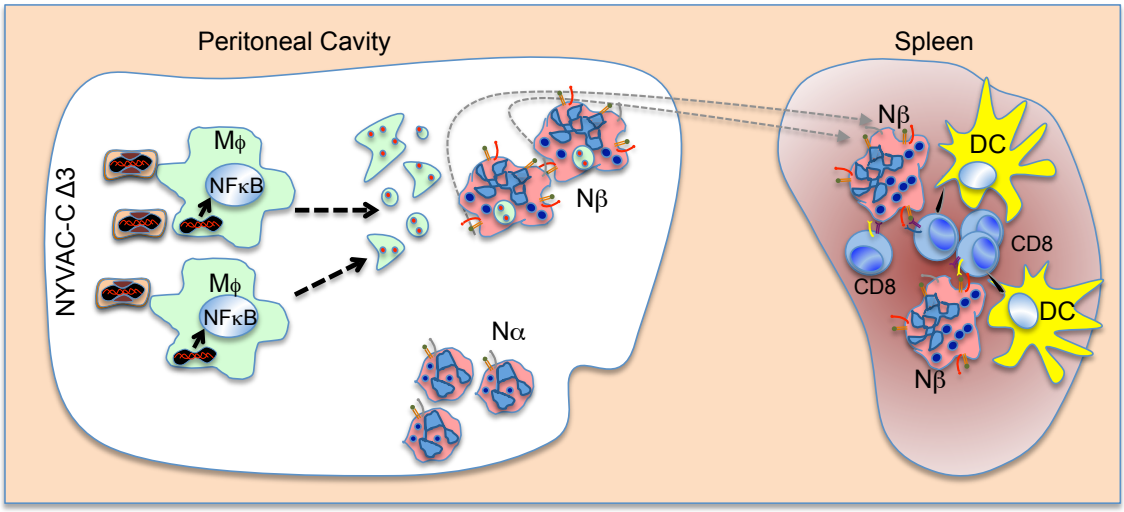

Figure 9. Model of neutrophil-dependent CD8 T cell activation

Apoptotic infected macrophages (Mϕ) are engulfed by neutrophils (Nβ) that cross-prime CD8 T cells with the help of dendritic cells (DC).

4.4 T cell responses to HIV

The attenuated vaccinia virus MVA, which lacks an anti-apoptotic gene (*F1L*), generates higher specific Gag-Pol HIV responses than the parental virus by inducing extensive apoptosis (Perdiguero et al., 2012). Neutrophil cross-priming of apoptotic peritoneal cells could explain why CD8 T cell responses to non-secreted Gag-Pol antigens were significantly higher in NYVAC-C Δ3- than in NYVAC-C-infected mice. In contrast to the induction of Gag and Pol responses, NYVAC-C Δ3 does not increase the CD8 T cell response to Env. This difference in antigen response probably depends

on extracellular secretion of Env compared to intracellular production of Gag-Pol. Our data suggest greater neutrophil involvement in the cross-priming of antigen from vaccinia virus-infected cells such as Gag and Pol than of Env secreted antigen.

The ability to generate high polyfunctional Gag- and Pol-specific CD8 T cell responses is an important immune property of the NYVAC-C Δ3 vector for future vaccine applications. Human HIV non-progressors preferentially maintain highly functional HIV-specific CD8 T cells (Betts et al., 2006). Gag and Pol, the best conserved HIV-1 proteins (Goulder and Watkins, 2008), can shift the CD8 T cell response from the variable Env epitope in the first years of HIV-1 infection (Yang et al., 2011a). In untreated chronic HIV-1-infected individuals, a Gag CD8 T cell response correlates with lower HIV viral loads (Kiepiela et al., 2007); furthermore, it correlates with decreased viremia in early HIV-1-infected patients with suspension of retroviral therapy (Yang et al., 2011a). A prophylactic vaccine that induces a Gag T cell response was recently shown to control simian immunodeficiency virus (SIV) infection (Iwamoto et al., 2014). Due to the limited vaccine effectiveness (31.2%) of ALVAC poxvirus in the RV144 phase III HIV/AIDS human clinical trial (Rerks-Ngarm et al., 2009) and to the promising results for MVA poxvirus in SIV-challenged primates (Barouch et al., 2013), new poxvirus vectors will be tested and NYVAC-C ΔA52RΔB15RΔK7R could be a valid vaccine candidate for prophylactic and therapeutic treatment of HIV.

4.5 NYVAC and NFκB inhibitors

We defined the biological effect of the deletion of three VACV inhibitors (A52, K7 and B15) in the NYVAC genome on the NFκB signaling pathway. These early proteins, members of the B cell lymphoma-2 family (Bcl-2) (Graham et al., 2008; Kalverda et al., 2009), are involved in suppression of Toll-like receptor (TLR)-induced host immune responses (Smith et al., 2013). Using single, double and triple deletion mutants, we demonstrated that A52, K7 and B15 do not act in synergy in their inhibition of the NFκB pathway; combined deletion of *A52R*, *K7R* and *B15R* genes is thus necessary for efficient triggering. With the generation of NYVAC-C Δ3 K7R-rev, we also determined that the presence of only one of the three inhibitory molecules is sufficient to abolish NFκB activation. It will be interesting analyze the role of these three proteins in the activation of TLR-dependent transcriptional factors such as IFN-regulatory factors (IRF) 3 and 7 and activator protein 1 (AP-1).

We defined that A46, another Bcl-2 protein involved in suppression of TLR-induced immune responses (Smith et al., 2013), induces cytokine/chemokine secretion that correlates with an increased HIV-specific T cell immune response. It has not been established whether the pro-inflammatory signals induced by NYVAC-C ΔA46R are NFκB-dependent or are mediated by distinct transcriptional factors. In the TLR2/TLR4-dependent pathway, the contributions of A46 and of C49, a newly defined NFκB inhibitor (Mansur et al., 2013), could be better determined by removing their genes in conditions of strong NFκB activation, such as those elicited by NYVAC-C Δ3 (Figure 10).

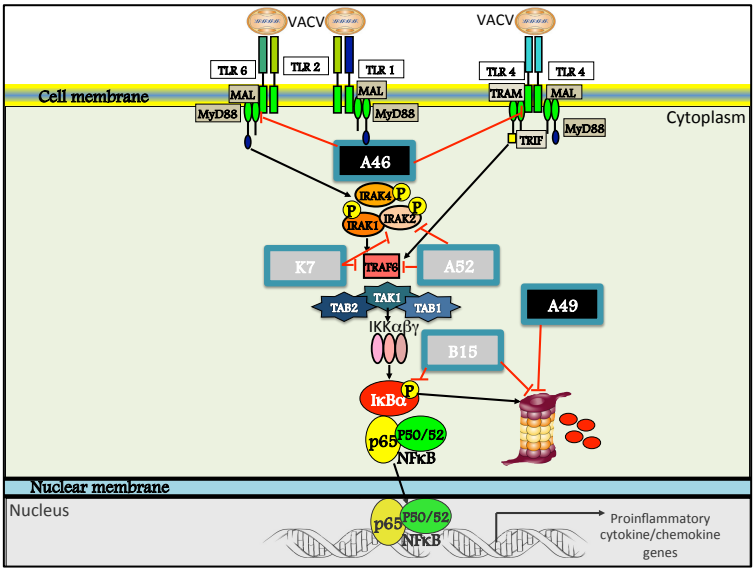

Figure 10. TLR pathway and NYVAC inhibitors. NYVAC inhibitors of TLR pathway, boxes with blue lines. NYVAC inhibitors of NFκB studied here, grey boxes.

4.6 Poxvirus promoter modification: sequence

In the second study, we focused on poxvirus promoter sequence modification to increase expression of the early heterologous antigen. We showed that compared to the early-late pS promoter, the late-early optimized (LEO) promoter increased GFP expression in the first hours post-infection; this increase in early antigen expression correlated with a significant enhancement of GFP-specific CD8 T cell responses in MVA-LEO-GFP-infected mice compared to those injected with MVA-pS.

Several recent studies report that new early promoters increase the expression of antigen under their transcriptional control as well as antigen-specific CD8 T cell responses (Baur et al., 2010; Isshiki et al., 2014; Wennier et al., 2013) when compared to p7.5, the first promoter described (Cochran et al., 1985), and to the widely used pS (Chakrabarti et al., 1997). All of these new promoters have early motif repetitions and have been compared with pS and p7.5, which have one early motif. Although the

advantage is evident for early antigen expression induced by promoters with more than one repetition, it was not known whether this antigen expression could be improved using a different promoter with one repetition. The LEO promoter has a single early motif, like the pS promoter, which increases antigen expression in HeLa cells when compared to p7.5 (Baur et al., 2010).

We demonstrate that the LEO promoter improves early antigen expression compared to pS; the LEO promoter sequence is based on our bioinformatics analysis of E1.1 promoter sequences, which are recognized more rapidly than those of E1.2. Although the E1.1 core promoter corresponds more closely to the consensus sequence than that of E1.2 (Yang et al., 2010b), whether the LEO promoter early sequence is better than that of pS remains to be defined due to differences in spacer length and late motif position. The generation of a new pS inverted (pS INV), in which the late pS sequence precedes the pS early motif and LEO spacer sequence (Figure 11A), and its comparison with LEO and pS promoters will help us respond to this question (Figure 11 B).

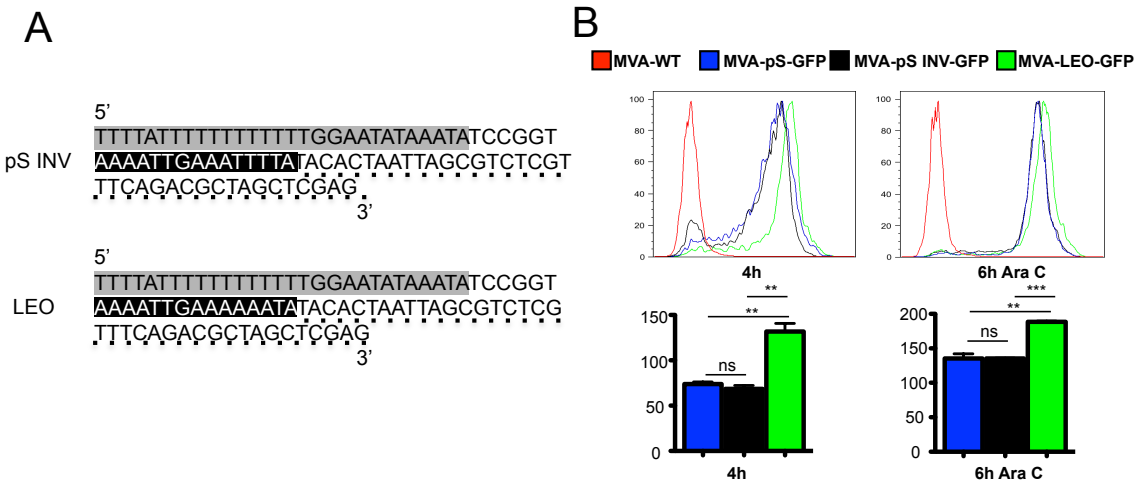

Figure 11. (A) Scheme of pS INV and LEO promoters. Late promoter element, grey box; early promoter motif, black box; spacer, dashed line beneath the sequence. **(B) GFP expression in HeLa cells infected with MVA-WT, MVA-pS-GFP, MVA-ps INV-GFP or MVA-LEO-GFP viruses.** Graphs show the *x*-fold increase in GFP median fluorescence intensity (MFI) of MVA-GFP compared to MVA-WT virus. Values shown as mean ± SEM. ** $p<0.01$, *** $p<0.001$.

We showed that the LEO promoter significantly increased GFP expression compared to pS INV, which differ in three early motif nucleotides (AAA to TTT); there were no differences between pS and pS INV. These results demonstrate that the new early motif sequence of the LEO promoter is more effective than the early motif in pS.

4.7 Poxvirus promoter modification: spacer

In the next study, we focused on the role of the poxvirus promoter spacer in the optimization of early antigen expression. We designed two new promoters with distinct spacer lengths, LEO99 and LEO160, and compared them with the previously reported LEO promoter (Di Pilato et al., 2013). Compared to LEO, the LEO99 and LEO160 promoters (with 99 and 160 nucleotide [nt] spacers) increased early antigen expression, demonstrating a positive correlation between spacer length and antigen expression.

The 50-nt window, from -70 to -20 nt upstream of the open reading frame, is the most frequent site for the promoter motif in early genes (Di Pilato et al., 2013). Our result suggests that VACV, whose early genes rarely have spacers longer than 70 nt, does not alone induce the highest possible gene expression. Viruses save on genome to gain replicative and genome stability advantages (Ojosnegros et al., 2011), which could explain how vaccinia viral genes maintain short spacers between the gene and their early promoter cores.

The DNA footprint of vaccinia RNA polymerase ternary complexes is less than 50 nt (Hagler and Shuman, 1992). The vaccinia virus early transcription factor (ETF) (Broyles et al., 1988) and RNA polymerase-associated protein (RAP94) (Ahn et al., 1994) mediate the promoter-RNA polymerase link. We hypothesized that spacers >50 nt would offer greater space to the transcription machinery, possibly accelerating gene expression. Further research is needed to determine which is the most appropriate spacer length to increase gene expression. Spacers with more than 99 nt offer advantages of early gene expression, although it remains unknown whether spacers with more or less than 160 nt are similarly advantageous.

Early gene expression mediated by promoter optimization is another strategy to increase CD8 T cell immunogenicity to a foreign antigen (Baur et al., 2010). Although VACV intermediate antigens are recognized preferentially by CD4 T cells (Yang et al., 2011b), we demonstrate that early antigen expression driven by the MVA-LEO160 early promoter can also positively influence antigen-specific CD4 T cell responses.

4.8 LACK-specific T cell responses

We demonstrated that MVA-LEO160 increases the CD4 and CD8 T cell response to the LACK *Leishmania* antigen compared to MVA-LEO; mice infected with MVA-LEO160-LACK presented higher numbers of antigen-specific polyfunctional CD4 and CD8 T cells compared to those infected with MVA-LEO.

The Th1:Th2 CD4 subtype ratio is crucial for leishmaniasis protection, as the Th2 response is associated to greater susceptibility to *Leishmania* (Launois et al., 2007) and the Th1 response protects against leishmaniasis infection (Darrah et al., 2010; Darrah et al., 2007). By increasing the number of IL-2$^+$IFNγ^+TNFα^+ CD4 Th1 cells, MVA-LEO160-LACK could be a valid vaccine candidate for leishmaniasis. MVA-LEO160-LACK also enhanced CD107a$^+$ memory CD4 T cells to LACK. These cells are resistant to HIV-1 infection, which implies that they could control the infection (Terahara et al., 2014); there is no evidence in the *Leishmania* model of a role for CD107a$^+$ CD4 T cells that correlates with protection from leishmaniasis.

Although several studies attempted to identify the phenotype and the role of CD8 T cells in natural *Leishminia* infection (Nateghi Rostami et al., 2010; Khamesipour et al., 2012) and after therapy (Papadogiannakis et al., 2010), it is not yet clear whether CD8 T cells have a positive or negative role in control of the disease. We defined LACK-specific CD8 T cells expressing IL-2, IFNγ, TNFα and CD107a, or IFNγ, TNFα and CD107a as the subsets most elicited by MVA-LEO-LACK and MVA-LEO160-LACK; both cell subsets were significantly higher in MVA-LEO160-LACK-injected mice. Polyfunctional CD8 T cells that express IL-2 and IFNγ induce Th1 polarization of CD4 T cells, limiting the natural Th2 response (Uzonna et al., 2004), and could thus have a function in *Leishmania* protection. CD8 T cells expressing IFNγ and TNFα could have a central role in control of the disease; by inducing macrophage activation and nitric oxide synthesis (Bogdan et al., 1990), both cytokines have a protective role against *Leishmania*. The CD8 T cell subsets elicited by MVA-LEO160-LACK also produced CD107a. CD107a$^+$ CD8 T cells correlate with necrosis intensity and lesion size in leishmaniasis (Santos Cda et al., 2013); if the excessive cytolytic activity of CD107a$^+$ CD8 T cells induces development of metastatic lesions (Launois et al., 2007), vaccine induction of these cells should be avoided.

CONCLUSIONS

5. Conclusions

1. The vaccinia virus (VACV) A52, B15 and K7 proteins are directly involved in inhibition of the NFκB signaling pathway, and the concomitant deletion of their genes is crucial for inducing robust activation of this pathway after NYVAC infection.

2. *In vivo* and *in vitro*, NYVAC-C-ΔA52RΔB15RΔK7R-dependent NFκB activation increases neutrophil migration compared to the parental NYVAC-C due to cyotokine/chemokine milieu generated at the infection site.

3. Nα and Nβ neutrophil subtypes induced by NYVAC-C-ΔA52RΔB15RΔK7R infection in mice show distinct antigen-presenting cell features; Nβ cells are implicated in CD8 T cell activation.

4. In mice, NYVAC-C-ΔA52RΔB15RΔK7R improves the magnitude and polyfunctionality of HIV Gag/Pol-specific CD8 T cell responses compared to the parental NYVAC-C, due to neutrophil migration.

5. The VACV late-early-optimized (LEO) promoter, generated based on bioinformatics analysis, increases early GFP antigen expression compared to the pS promoter, due to an improved temporal early promoter sequence.

6. The enhanced GFP expression induced by recombinant MVA-LEO virus correlates with an increase in magnitude and polyfunctionality of GFP-specific CD8 T cell responses in mice.

7. The modifications in the spacer length of the LEO promoter demonstrate that a longer promoter spacer increases early antigen expression under LEO transcriptional control.

8. In mice, MVA-LEO160-LACK enhances *Leishmania* LACK-specific CD4 and CD8 T cell responses compared to MVA-LEO-LACK, which confirms the positive correlation between antigen expression and T cell responses to antigen.

CONCLUSIONES

6. Conclusiones

1. Las proteínas A52, B15 y K7 del virus vaccinia (VACV) están directamente involucradas en la inhibición de la vía de señalización de NFκB y la deleción concomitante de sus genes es crucial para inducir una activación robusta de esta vía tras la infección por NYVAC.

2. *In vivo* e *in vitro*, la activación de NFκB inducida por NYVAC-C-ΔA52RΔB15RΔK7R aumenta la migración de los neutrófilos en comparación con el virus NYVAC-C debido a las citocinas/quimiocinas producidas en el lugar de infección.

3. Los subtipos Nα y Nβ de neutrófilos, inducidos tras la infección en ratón con NYVAC-C-ΔA52RΔB15RΔK7R, muestran distintas características de células presentadoras de antígeno; los Nβ están implicados en la activación de células T CD8.

4. NYVAC-C-ΔA52RΔB15RΔK7R mejora en ratón la magnitud y la polifuncionalidad de la respuesta específica de células T CD8 frente a los antígenos de VIH Gag y Pol en comparación con el virus parental NYVAC-C debido a la migración de neutrófilos.

5. El promotor tardío-temprano optimizado (LEO) de VACV, generado tras el análisis bioinformático, aumenta la expresión temprana del antígeno GFP en comparación con el promotor pS gracias a una optimización de la secuencia temprana del promotor.

6. La expresión mejorada de la GFP inducida por el virus recombinante MVA-LEO se correlaciona en ratón con el aumento de la magnitud y polifuncionalidad de las respuestas de células T CD8 específicas frente a la GFP.

7. Las modificaciones en la longitud del espaciador del promotor LEO demuestran que un espaciador más grande aumenta la expresión del antígeno.

8. El virus MVA-LEO160-LACK mejora en ratón las respuestas de células T CD4 y CD8 frente al antígeno LACK de *Leishamina* en comparación con MVA-LEO-LACK, lo que confirma la correlación positiva entre la expresión del antígeno y la respuesta de células T hacia este mismo antígeno.

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APPENDIX